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Review

Analytical and compositional aspects of isoflavones in food and their biological effects

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This paper provides an overview of analytical techniques used to determine isoflavones (IFs) in foods and biological fluids with main emphasis on sample preparation methods. Factors influencing the content of IFs in food including processing and natural variability are summarized and an insight into IF databases is given. Comparisons of dietary intake of IFs in Asian and Western populations, in special subgroups like vegetarians, vegans, and infants are made and our knowledge on their absorption, distribution, metabolism, and excretion by the human body is presented. The influences of the gut microflora, age, gender, background diet, food matrix, and the chemical nature of the IFs on the metabolism of IFs are described. Potential mechanisms by which IFs may exert their actions are reviewed, and genetic polymorphism as determinants of biological response to soy IFs is discussed. The effects of IFs on a range of health outcomes including atherosclerosis, breast, intestinal, and prostate cancers, menopausal symptoms, bone health, and cognition are reviewed on the basis of the available *in vitro*, *in vivo* animal and human data.

Keywords: Bioavailability / Biological effects / Cancer / Databases / Isoflavones

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1 Introduction

Over the last two decades the biological effects of bioactive compounds from plants have received considerable attention with particular interest of soy isoflavones (IFs) in relation to human health. Several epidemiological studies have correlated consumption of soy IFs with multiple beneficial effects on atherosclerosis, breast and prostate cancers, menopausal symptoms, and bone density. Also their hormonal and nonhormonal properties indicated potential for beneficial health effects. Thus, their biological effects have been extensively studied in *in vitro* systems, laboratory animals and animal models of human diseases, and in humans. Simultaneously, methods for analyzing the content of soy

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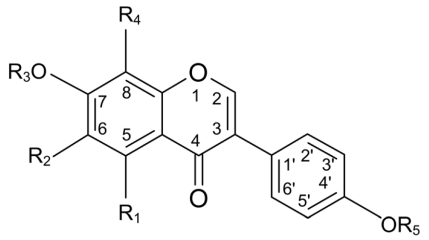
Abbreviations: ADME, absorption, distribution, metabolism, and excretion; **cAMP,** cyclic 3′,5′-adenosinemonophosphate; **CRC,** colorectal cancer; **DHD,** dihydrodaidzein; **eNOS,** endothelial nitric oxide synthase; **ER,** estrogen receptor; **FFQ,** food frequency questionnaire; **HUVEC,** human umbilical vein endothelial cells; **ICAM,** intercellular adhesion molecule; **IC_{50},** half maximal inhibitory concentration; **IF,** isoflavone; **iNOS,** inducible nitric oxide synthase; **LDL,** low-density lipoproteins; **MCP,** monocyte chemoattractant protein; O-**DMA,** *O*-desmethylangolensin; **OH,** hydroxy; **PGI_2,** prostacyclin; **PKA,** protein kinase A; **PSA,** prostate specific antigen; **SGLT1,** sodium-dependent glucose cotransporter; **TNF,** tumor necrosis factor; **TVP,** textured vegetable protein; **VCAM,** vascular cell adhesion molecule

IFs in plants, foods, and other biological material have been developed. This enabled investigation of their metabolism and kinetics on the one hand, and generation of databases on the content of soy IFs in food items on the other hand, allowing the generation of intake data in different populations. In the last decade, several reviews have collated evidence on health effects [1–13]. In this review, we present the current knowledge on soy IFs including analytical aspects as well as information on IF databases, intake estimations, metabolism, kinetics, and estrogenic action. Further, the antioxidant activity, cardiovascular effects, genetic polymorphism in response to IFs, as well as effects on cancer and menopausal symptoms, bone health, and cognition are discussed. This interdisciplinary paper reflects the multidisciplinary approach directed toward elucidation of the biological effects of soy IFs in order to create a basis for dietary improvement of human health.

2 Sources of IFs and IF contents in foodstuffs

IFs are diphenolic compounds present mainly in plants of the Leguminosae family. Highest concentrations of IFs occur in soybeans, red clover, and kudzu root. Soybeans contain predominantly daidzein and genistein, and, in lower concentration, glycitein. The main IFs in red clover are formononetin and biochanin A and the principal IFs in kudzu root are puerarin and daidzein. In unprocessed soybeans and red clover, IFs occur predominantly as 7-*O*-β-glucosides and as 6″-*O*-malonyl-7-*O*-β-glucosides. The structures of the main IF aglycones are given in Fig. 1.



Compound	R1	R2	R3	R4	R5
Daidzein	H	H	H	H	H
Glycitein	H	OCH_3	H	H	H
Genistein	OH	H	H	H	H
Formononetin	H	H	H	H	CH_3
Biochanin A	OH	H	H	H	CH_3
Puerarin	H	H	H	Glc	H

Figure 1. Structures of IF aglycones. Glc: glucose. IF-7-*O*-β-glucosides: R_3 = glucose.

2.1 Content of IFs in soy foods

The main food sources of IFs are traditional soy foods, new generation soy products, and commonly consumed foodstuffs in the production of which soy flour or soy protein isolates are used. IF concentrations reported for the former two food groups cover a wide range which is, in part, due to the high natural variability of the IF content in soybeans. Concentrations of total IFs (sum of daidzein, genistein, and glycitein in aglycone equivalents) determined in different types of soy flour range from 60 to 265 mg/100 g [14]. Tofu has been reported to contain between 5.1 and 64 mg/100 g total IFs [14], soy milk between 1.3 and 21 mg/100 g, miso between 23 and 126 mg/100 g [14, 15], natto between 20 and 124 mg/100 g [16, 17], and tempeh between 6.9 and 63 mg/100 g [14]. Concentrations of IFs in soy sauce are generally low (0.1–2.3 mg/100 g) [14].

New generation soy foods are IF sources of increasing popularity in the Western population. Soy burgers (0.1–26 mg/100 g total IFs in aglycone equivalents ([18], Database, Ritchie)), soy yogurts (1.6–11.8 mg/100 g ([18], Database, Ritchie)), soy milk drinks (1.0–11 mg/100 g [14, 18]), and soy cheeses (2.3–33 mg/100 g (Database, Ritchie, [19])) are typical examples. Commonly and frequently consumed foodstuffs containing variable, but generally low concentrations of total IFs (usually <2 mg/100 g ([20, 21], Database, Ritchie)) are bakery products like bread and muffins, meat products like sausages and canned food items like tuna or meatless chili in the production of which soy flour (bakery products) or soy protein (meat products, chili) may have been added. Infant formulas, on the other hand, may contain high concentrations of total IFs (up to 31 mg/100 g instant product [14]). However, highest IF concentrations are found in nutritional supplements which may consist of up to 40% IFs [22]. IF supplements are commonly manufactured from extracts of soy and red clover and in some cases also from kudzu root.

2.1.1 Natural variability

The concentration of IFs in soy beans is highly variable [23] for several reasons. The influence of the soybean cultivar on the IF concentration has been investigated and found to be a major factor for the variability [24–33]. Additional parameters of great impact are the crop year [26, 29–31, 34] which, reflecting the weather conditions (precipitation, sunshine, light, temperature) at the time of cultivation, has been attributed an even higher influence than the cultivar [29, 30, 35], the sowing date [24], the growing location [29], and country of origin (reflecting different climatic, geologic, and agricultural conditions) [19, 35, 36], the conditions during seed development (temperature, carbon dioxide level, drought) [37], and the maturity stage of the soybean seed [32]. Total IF concentrations (sum of daidzein, genistein, and glycitein in aglycone equivalents) determined in raw, mature soybeans range from 18 to 562 mg/100 g [26, 29]. Minor sources containing IFs in the ppb- to low-ppm range are other legumes, fruits, and vegetables [14, 15, 38]. Prolonged storage at ambient temperature has been shown to decrease malonyl glucosides and concomitantly to increase glycosides and aglycones. The total IF

concentration decreased only slightly upon storage for 3 years [26, 29].

2.1.2 Influence of processing

The use of different processing conditions contributes to the variability of IF concentrations in processed soy foods. Studies have been undertaken to investigate the influence of the processing conditions on the IF content and IF pattern of soybeans [32, 39–45], soy milk [40, 41, 46], and tofu [40, 41], to assess the impact of the soybean variety and soybean tissue in the production of tempeh [47], to look into the role the type of soybean (whole *vs.* defatted) plays in the manufacture of soy sauce [48, 49], and to determine the influence of different starter organisms and fermentation temperatures in the production of black bean koji [50].

The variation of total IF aglycone levels in different brands of tofu products amounted to 129% for packaged tofus from the USA, to 151% for Indonesian tofus and to 178% for tofus purchased in Australia [19, 51]. IF contents of different batches (purchased during 2 years) of the same brand varied by up to 28% [51].

Soy milks of different types had significantly different IF levels and genistein to daidzein ratios [16]. In addition, brand- to brand differences were up to 5-fold for direct extract soy milks and differences of total IF levels in items of the same brand purchased over the period of 6 months varied by as much as 60%. The same authors also investigated the variability of the IF contents in 30 separate samples of two soy protein isolates purchased over a 3-year period and reported that total IF levels varied by 200–300% over 3 years [16]. In general, the high variability of IF contents in soy protein concentrates [52], isolates, and textured vegetable protein (TVP), ingredients used in the production of new generation soy foods, and the use of different formulations are the reason for the wide range of IF levels in "modern" soy foods. In the case of dietary supplements, use of different plant parts for extract preparation introduces a further source of variation [53]. Soy germ contains 6–10 times higher total IF levels than soy cotyledons [25] and is usually rich in malonyl glycitin and glycitin (up to 50%). Genistein conjugates, on the other hand, occur mainly in the cotyledons (>50%) and only to about 10% in soy germs [25]. Likewise, highest IF concentrations are found in red clover leaves, followed by stem and inflorescences. However, both the IF pattern and the IF distribution in different plant parts are also influenced by the stage of maturity [54].

In addition to covering a wide range of IF concentrations, different soy foods and IF supplements have a variable IF conjugation pattern. Upon heat treatment [55, 56] malonyl glucosides are, depending on the conditions, degraded to acetyl glucosides, glycosides, and/or aglycones (Fig. 2). The presence of acetyl glucosides is therefore an indicator of thermal processing. However, acetyl glucosides can themselves be degraded to glycosides and aglycones. Similarly, fermented foodstuffs like soymilk, tempeh, miso, or koji contain comparatively high levels of free aglycones due to the action of β-glucosidases from the fermentation organisms [47, 50, 57–60]. The impact of different processing methods (Fig. 2) on the IF conjugation profile has been summarized in [61, 62] and can be studied in all papers reporting nonhydrolytic extraction of IFs from foodstuffs.

2.2 IF databases

One of the main reasons for creating IF databases is the provision of IF contents in a wide range of foodstuffs to enable IF intake assessment. Early created literature compendia and databases [21, 63, 64] helped to identify IF containing foods, to provide a first overview of IF contents in foodstuffs and to prioritize future analyses by pointing out areas where data were lacking. Nevertheless, most IF databases were created for dietary IF intake assessment in epidemiological studies, sometimes in conjunction with a food frequency questionnaire (FFQ) [20, 21, 65, 66], sometimes on the basis of data obtained from national food consumption studies [67–71]. Comprehensive databases for nutrition research are currently being developed ([72], Scalbert, A., personal communication, French database on polyphenols, 2007) or updated [14]. A review summarizing the existing phytoestrogen databases and discussing their applications, advantages and limitations are given by Schwartz *et al*. (Inventory of phytoestrogen databases, *Food Chem*. 2009, *113*, 736–747).

Whereas early databases covered only a small number of foods (<150), some of the databases created in and after 2003 provided data for several hundreds of different or differently processed foods [67, 68]. In 2006, a free access internet deployed IF database comprising about 6000 foods was developed ([69], Ritchie, M. R., Phytooestrogen database, http://medicine.st-andrews.ac.uk/research/docs/ritchie/, accessed on 5.4.2007). The data sources used in these databases were manifold: Peer-reviewed literature, chemical analysis, recipe calculation, calculation based on nutrient information from supermarket chains or from the manufacturers, estimation based on the IF content of similar foods and other databases.

In most databases, the IF contents are not aggregated and given in aglycone equivalents on a wet weight basis. With the exception of [66, 69] which give only sum values of total IFs compounds are listed separately in the investigated databases. Daidzein and genistein are covered in all databases, whereas glycitein is listed only in the BASIS databases [72], the USDA database [14], and the database by Thompson *et al*. [71]. Values for formononetin and biochanin A can be found in ([14, 20, 21, 63, 67, 71, 72], Scalbert, A., personal communication, French database on polyphenols, 2007).

The individual databases differ greatly in the amount of additional information presented. Databases which are cur-

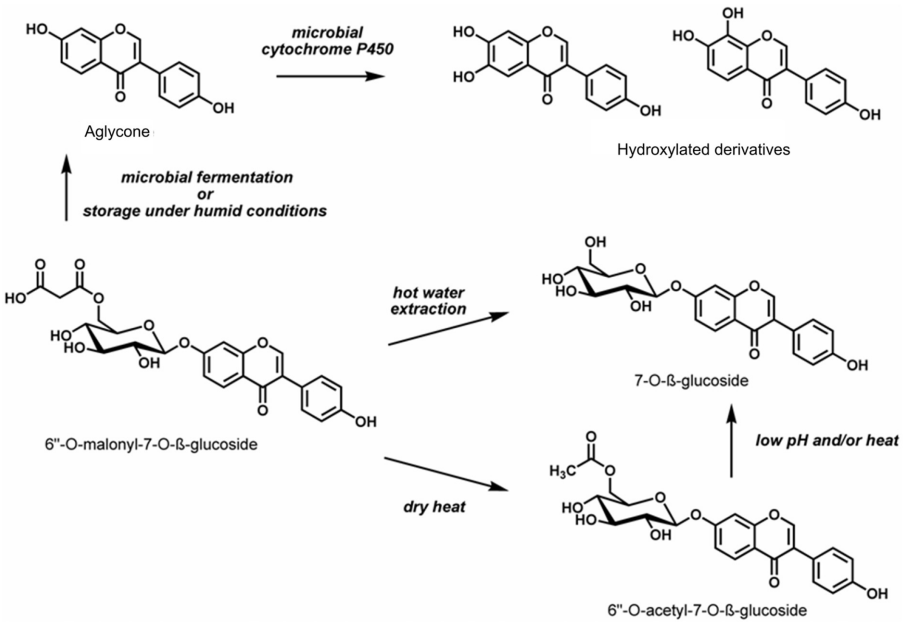


Figure 2. Chemical forms of the IFs (daidzein is shown as an example) in soy food and their modification during cooking and processing.

rently being developed ([72], Scalbert, A., personal communication, French database on polyphenols, 2007) or updated [14] provide the greatest amount of additional information on the food plant, the processing method, and on the analytical method which makes them most suited for nutrition research.

A topic of increasing importance in connection with IF databases is the quality control of the data. In some databases, data entries are accompanied by quality codes, in others, quality control is performed prior to inclusion of data into the database. Quality control according to an expert quality assessment scheme (considering sampling plan, sample handling, analytical method, analytical quality control, and number of samples ([72, 73, 74], Plumb, J., personal communication, EuroFIR BASIS database)) was carried out in ref. [14, 66, 68, 70, 72].

Of the 15 databases mentioned in this review, 5 are not yet or not any more available to the public. These are the Venus database [67] (project finished), the IF part of the Finnish national food composition database [68] (only for internal use and for selected customers), the original BASIS database (presented on CD-ROM and currently being expanded and updated in the form of the EuroFIR BASIS database), the EuroFIR BASIS database [72], and the French database on polyphenols (Scalbert, A., personal communication, French database on polyphenols, 2007) (both under construction).

The fact that a lot of work has already been put into creating IF databases must not belie that there are several difficulties associated with their establishment. One of the main problems is the high variability of IF contents in foods of the same kind which is due to the natural variability, the variability introduced by processing and, in part, due to use of different analytical methodologies. In addition, many values were created by analyzing convenience samples, *i.e.*, single samples purchased in low quantities at convenient locations without proper sampling plan which gives rise to unrepresentative data. As mentioned above, peer-reviewed literature is one of the main data sources for IF databases. However, data reported in papers describing the development of analytical methods are often of low quality due to insufficient food description and lacking sampling plan. On the other hand, authors investigating the natural variability or giving IF contents for various foodstuffs sometimes use outdated or not validated analytical methods. In some databases, low quality data are not included in the first place. Yet, the line between insufficient and just acceptable data is hard to draw. For instance, data which are insufficient due to lacking sampling plan may be of use when data are aggregated. Therefore, some databases include low quality data, give them a low confidence code and use them for data aggregation. In general, data aggregation is a good way of minimizing the impact of the natural variability on the mean value.

To sum up, IF databases are useful tools for identifying food sources of IFs, for providing the range in which IFs occur in foodstuffs and for rough estimations of the dietary intake of IFs in epidemiological studies [75]. In addition, the EuroFIR BASIS database which contains also information about biological effects can be used for investigating food and health relationships, for risk assessment and for the development of novel foods [72]. IF databases should not be used for calculation of exposure in human intervention studies due to the high biological variation of IF con-

tents in foodstuffs of the same type. Therefore, the concentration of IFs in test foods must be determined by chemical analysis in these studies [16, 75].

3 Determination of IFs in foods and dietary supplements

The process of obtaining IF contents in foodstuffs and nutritional supplements includes sampling, sample handling, sample preparation, analytical measurement, and data evaluation. Sampling (collection of sample units which, together, form the sample which should be representative for the population of a food [76]) is the factor with the greatest impact on the representativeness of the obtained data. Ideally, sampling should be performed as described in [73, 76]. Sample handling (or sample pretreatment) starts after acquisition of the sample and ends with the preparation of a homogenous sample for analysis. The actual analytical procedure comprises two parts: sample preparation (or sample work-up) and analytical measurement. The main emphasis in this review will be on sample preparation because the analytical methods have recently been summarized in comprehensive reviews.

3.1 Sample preparation

Basically, sample preparation techniques can be divided in hydrolytic and nonhydrolytic methods. The advantages of hydrolytic methods are that only aglycone and, depending on the extent of hydrolysis, glucoside standards are required and that simpler chromatograms are obtained (allowing shorter analysis times). Limitations are stability problems of some IFs in acidic and alkaline solution, incomplete hydrolysis under mild conditions, loss of information on the conjugation pattern, longer sample preparation times for hydrolysis of extracts and the dependence of the IF content obtained by enzymatic hydrolysis on the extraction efficiency. Hydrolysis of conjugated IFs can be achieved by acid hydrolysis, *e.g.* [19, 33, 51, 77–86], basic hydrolysis, *e.g.* [87–89], or enzymatic hydrolysis, *e.g.* [15, 20, 38, 71, 90–94]. The most commonly employed method is acid hydrolysis. It is capable of cleaving both glucosidic bonds and ester linkages. However, the conditions have to be carefully chosen in order to achieve quantitative cleavage without degradation of analytes. Several papers reporting the use of mild conditions (refluxing in 0.3–1.2 M HCl, for 15–120 min), *e.g.* [33, 54, 79, 81, 82, 84, 85] or in 80% aqueous methanol adjusted to pH 3 with acetic acid [80], more drastic conditions (refluxing in 2 M HCl for 1–6 h), *e.g.* [19, 51, 78, 86], and drastic conditions (refluxing in 3 M HCl for 40 min) [77] have been published. Still, it is possible that cleavage was not quantitative under mild conditions and that genistein was partly degraded using 2 and 3 M HCl. A compromise has been suggested by Penalvo *et al.* [83] who hydrolyzed with 1 M HCl at 80°C for one hour, thereby cleaving malonyl- and acetyl glucosides to glucosides, and quantitated both glucosides and aglycones. However, this approach requires both aglycone and glucoside standards. Enzymatic hydrolysis, though applied by fewer authors, has been used to determine the IF contents in a wide range of foodstuffs and dietary supplements [15, 20, 38, 71, 90–97]. Most analytical data in the database established by Ritchie *et al.* ([69], Ritchie, M. R., Phytooestrogen database, http://medicine.st-andrews.ac.uk/research/docs/ritchie/, accessed on 5.4.2007) are from [15, 20, 38, 92, 95, 96].

Nonhydrolytic methods provide information on the IF conjugation pattern. With the introduction of new or newly developed extraction methods (accelerated solvent extraction [27, 98–100], supercritical fluid extraction [101], and sonication [100, 102, 103]), the sample preparation time has been reduced so that nonhydrolytic sample preparation can be performed faster. The limitations of direct extraction and analysis of the extracts are that malonyl and acetyl glucoside standards are required (which are of limited stability [104, 105]) unless calibration functions for glucosides are used to quantitate malonyl and acetyl forms after correction for the molecular weight difference, that more complex chromatograms are obtained (requiring longer HPLC-run times), that analysis by GC is not possible and that some minor forms of malonyl glucosides are formed during the extraction [106]. Extraction can be carried out by different techniques using different solvents: stirring at room temperature (to avoid degradation of native forms) [17, 26, 29, 31, 58, 106–112] or at elevated temperature (to enhance the extraction efficiency) [18], refluxing [16, 24, 113], extraction by sonication [100, 102, 103], microwave assisted extraction [114], accelerated solvent extraction [27, 98–100], supercritical fluid extraction [101], and SPE [115, 116]. Different solvents containing different percentages of water have been employed with [26, 29, 58, 107–112] or without addition of acid [17, 31, 106].

Several papers reporting attempts at finding the optimum extraction solvent, temperature, and hydrolysis conditions have been published [19, 57, 62, 77, 81, 83, 86, 87, 106, 117, 118]. However, for both hydrolytic and nonhydrolytic methods optimum extraction of analytes from different food matrices requires different extraction conditions. These have to be optimized for each analyte in each food matrix. It is therefore important that, after having developed a method, authors test the applicability of the method to analysis of other food matrices.

3.2 Analytical techniques

The most commonly reported analytical methods in IF analysis are HPLC coupled with UV, electrochemical or MS detection and GC coupled with MS. Comprehensive reviews discussing these chromatographic and nonchroma-

tographic methods (CZE, immunoassay) have been published [61, 119 – 126].

3.3 Evaluation of available data and recommendations

The natural variability of the IF contents in soybeans, the variability introduced by different processing conditions and use of different formulations, the limited comparability of the values obtained by different analytical methods and lacking information for some of the values reported in the literature pose great problems in the establishment of IF databases.

In many cases, the data quality could be considerably improved if authors provided more information on the sample itself (plant part and part of the food analyzed, whole or edible portion), maturity, country of origin, cultivar, region, season, year, processing method, product specific information like brand name and manufacturers, on the sampling procedure (sampling plan, time and region, sample size, number of sample units), and on sample handling. In addition, details about the analytical method and the validation of the analytical method should be given. For reasons of comparability, IF contents should be given on the same weight basis (preferably wet weight) and be expressed in aglycone equivalents.

It is seldom that all the required information is given in a paper. Food description, sampling, and sample handling are often neglected in method development papers. Likewise, some method development papers describe the testing of different extraction or hydrolysis conditions but neglect to validate the optimum method or to comment on its validation. In addition, papers concentrating on providing IF contents in foodstuffs or on determining the natural variability in foods of the same kind often give only limited information on the analytical method. However, this information would greatly facilitate the comparison of results obtained by different methods.

4 Dietary intake of IFs

4.1 IF intake in Asian populations

In Asian countries, fermented soy products such as tempeh, miso, or natto are part of the traditional diet. This leads to a mean daily IF intake of about 8 – 50 mg (expressed as aglycone equivalents) in Asian countries. The mean daily intake among older Japanese adults ranges from 25 to 50 mg. According to the National Nutrition Survey of 2002 in Japan, soy IF intake from soy foods fell in the range of 18 and 64 – 76 mg/day for the 50th and the 95th percentile [127 – 131]. Intake in Korea was estimated as 14.9 mg/day based on data from the Korean National Nutrition Survey conducted in 1995 [130]. The mean IF intake of women in China was found to be 25.4 mg/day, whereas the intake of Hong Kong Chinese women was found to be lower (7.8 ± 5.6 mg/day) and the intake of Singapore Chinese adults was found to be higher (61 mg/day) [132, 133]. The mean plasma IF concentration (sum of daidzein, genistein, and equol) in Japanese women and men consuming a traditional diet was 874 and 806 nmol/L, respectively [134, 135].

4.2 IF intake in Western populations

Intakes of IFs in Western populations are lower than in Asian populations since soy is not commonly consumed in Western diets. In Table 1, an overview is given of the IF intake studies which were performed in Western (European and North-American) countries [65, 68, 69, 136 – 145]. Furthermore, the resulting plasma concentrations are shown. Up to now data on IF intakes in Eastern Europe do not exist.

The dietary intake of IFs in Western countries is only less than one to several mg/day with plasma IF concentrations in the lower nanomolar range. The differences between the intake values obtained in the different studies are likely to be due to the different databases of IF contents in foods used by the different researchers and due to the different diets in the countries. The latter is probably less important, as the difference between the intakes calculated for the six studies performed in the UK is already a factor of 12.

4.3 Dietary intake of IFs by special subgroups

4.3.1 IF intake by vegetarians, vegans, and soy-consumers

The IF intake by vegetarians, vegans, and soy-consumers is expected to be higher than that of the whole population, mainly due to the higher intakes of soy and soy products. In Table 2, an overview of the estimated intakes is presented. The intake of the vegetarians and soy-consumers (3 – 12 mg/day) is still low compared to IF intakes in Asian populations (15 – 60 mg/day). The Western vegan mothers are the only adult subgroup which has IF intakes higher than Asian people [69, 142, 144, 146, 147].

4.3.2 IF intake by infants

In infant formulas based on cow's milk and in human breast milk, concentrations of IFs range between 0 and 50 μg/L (see Table 3). The highest concentrations are found in the breast milk of mothers following a vegetarian or vegan diet as well as in cow's milk. These concentrations are negligible low compared to the IF concentrations present in soy-based infant formulas (see Section 2.1). Soy-based infant formulas are used if children are allergic or intolerant to cow's milk or if parents chose to feed their children a vegan diet. Approximately 25% of the bottle-fed children in the US receive soy-based diet. The daily intakes of IFs in infants fed soy-based infant formulas, cow's milk infant formulas and breast milk are summarized in Table 4 [110, 143, 147 – 150, 527 – 529].

Table 1. Isoflavone intake by adult populations in Western countries

Reference	*N*	Subjects	Country	Mean IF intake *per* day (mg)	Food consumption data	Primary sources	Resulting mean plasma concentrations (nmol/L)
Horn-Ross *et al.* [139]	447	Women, 50 – 79 year	USA	2.87	FFQ, IF concentrations based on own database (Horn-Ross *et al.* [20])	Traditional soy-based foods and hidden sources of soy, *e.g.*, added soy protein isolate, concentrate or flour such as doughnuts or white bread	n.a.
de Kleijn *et al.* [65]	964	Postmenopausal women	USA	0.76 ± 4.35	FFQ, IF concentrations based on scientific literature	Beans, peas, tea, coffee, and nuts	n.a.
Rupp *et al.*, [143]	–	Whole population	Switzerland	1.7	Calculation with data from soybeans used to produce food products (traditional soy-based foods not included)	–	n.a.
Horn-Ross *et al.* [138]	2882	Women, 35 – 79 year	USA	3.3	FFQ, IF concentrations based on own database (Horn-Ross *et al.* [20])	Tofu, doughnuts, soy, milk, white bread	n.a
Keinan-Boker *et al.* [140]	35 955	Whole population, 35 – 74 year	10 European countries (DK, F, GER, GR, I, N, ESP, SW, NL, UK)	<2	IF concentrations based on USDA-Iowa State University database including only soy foods	Dairy substitutes, beans, sprouts	n.a.
Keinan-Boker *et al.* [141]	17 140	Women, 50 – 69 year	NL	0.88	FFQ, IF concentrations based on scientific literature	Beans, peas, nuts, grain products, coffee, tea, and soy products	n.a.
Valsta *et al.* [68]	2862	Whole population, 24 – 64 year	Finland	0.79 ± 067	24 h recall, IF concentrations based on the Finnish National Food Composition Database (Fineli$^{\text{®}}$)	Hidden sources of soy, *e.g.*, in meat and bakery products	n.a.
Van Erp-Baart *et al.* [144]	1379	Whole population, 18 – 64 year	Ireland	0.73 ± 1.77	7-day record, IF concentrations based VENUS database	n.g.	n.a.
Van Erp-Baart *et al.* [144]	1513	Whole population, up to 94 year	Italy	0.55 ± 1.51	7-day record, IF concentrations based VENUS database	n.g.	n.a.
Van Erp-Baart *et al.* [144]	335	Whole population, 40 – 64 year	UK	0.70 ± 1.04	7-day record, IF concentrations based VENUS database	n.g.	n.a.
Van Erp-Baart *et al.* [144]	4085	Whole population, 1 – 97 year	NL	0.91 ± 1.90	2-day record, IF concentrations based VENUS database	n.g.	n.a.
Clarke and Lloyd [145]	–	–	UK	3.3	Calculation using data from the FSA-total diet study, IF concentrations determined by analysis of 20 complete total diet study sample sets from 1998	Bead, meat products, and cereals	n.a.
Heald *et al.* [137]	203	Men, 50 – 74 year	UK	1.0 (median)	FFQ, IF concentrations based on scientific literature	Traditional soy-based foods and hidden sources of soy, *e.g.*, added soy protein isolate, concentrate or flour	196 (median)
Ritchie *et al.* [69]	19	Whole population, 19 – 76 year	UK	4.5 ± 4.89	7-day record, IF concentrations based on scientific literature	Soy milk, yogurt, and bread	n.a.
Bhakta *et al.* [136]	50	Women, 25 – 75 year	UK	0.37 ± 0.18	Monthly 24 h recalls for 1 year, IF concentrations based on scientific literature	Bread	25.8 ± 22.1
Mulligan *et al.* [142]	11 843	Whole population	UK	0.56 ± 0.46	7-day record, IF concentrations based on scientific literature	Bread, vegetable, and meat products	n.a.

n.a., not assessed, n.g. not given.

Table 2. Isoflavone intake by vegetarians, vegans, and soy-consumers in European countries

Reference	*N*	Subjects	Country	Mean IF intake *per* day (mg)	Food consumption data	Primary sources
Clarke *et al.* [146]	35	Vegetarians	UK	12	7-day duplicate diet analysis	Traditional soy-based foods and hidden sources of soy, *e.g.*, added soy protein isolate, concentrate, or flour such as doughnuts or white bread
Ritchie *et al.* [69]	10	Vegetarians, 21 – 56 year	UK	7.4 ± 3.1	7-day record, IF concentrations based on scientific literature	Soy milk, meat-substitute foods containing TVP and soy protein isolate, soy mince, and bakery products
Friar and Walker [147]	11	Vegan breast-feeding mothers	UK	75	Duplicate diet analysis	
Van Erp-Baart *et al.* [144]	42	Soy-consumers	Ireland	6.0 ± 8.1	7-day record, IF concentrations based VENUS database	n.g.
Van Erp-Baart *et al.* [144]	15	Soy-consumers	UK	3.2 ± 4.0	7-day record, IF concentrations based VENUS database	n.g.
Van Erp-Baart *et al.* [144]	85	Soy-consumers	NL	11.1 ± 6.7	2-day record, IF concentrations based VENUS database	n.g.
Mulligan *et al.* [142]	371	Soy-consumers	UK	7.1 ± 10.9	7-day record, IF concentrations based on scientific literature	Soy-based dairy products, vegetable dishes, and bread

n.g., not given.

Table 3. Total IF concentrations in breast and cow milk

Reference	Milk	IF concentration (μg/L)
Franke *et al.* [524]	Breast feeding: mothers' diet Chinese ($n = 1$)	35
King *et al.* [525]	Cow's milk	50 – 350
Friar and Walker [147]	Breast feeding: mothers' diet	
	Omnivorous ($n = 14$)	0 – 2
	Vegetarian ($n = 14$)	1 – 10
	Vegan ($n = 14$)	2 – 32
Setchell *et al.* [149]	Breast feeding: mothers' diet	
	Omnivorous ($n = 9$)	5.6 ± 4.4
Franke *et al.* [524]	Breast feeding: mothers' diet	
	Omnivorous ($n = 1$) challenged with 37 mg IFs	52
Irvine *et al.* [150]	Breast feeding: mothers' diet	
	Omnivorous ($n = 11$)	Below LOD (< 0.05 mg/L)
	Cow-based infant formula	Below LOD (< 0.1 mg/g)
Antignac *et al.* [526]	Cow's milk	5 – 32
Franke *et al.* [527]	Breast feeding: mothers' diet	
	Omnivorous ($n = 7$)	1.3 ± 0.6
	Omnivorous ($n = 7$) challenged with 55 mg IFs	18.5 ± 5.0

Infants' daily intakes of IFs from human breast milk or cow's milk formulas range between 0.005 and 0.01 mg/day which is low when compared with the amounts provided by soy-based infant formulas (6 – 47 mg/day). Furthermore, IFs are predominantly found as glucuronide conjugates in breast milk, whereas they occur mainly as glycosidic conjugates in soymilk [148]. Up to date it is not known if these compositional differences influence the bioavailability. Nevertheless, it is doubtful that the small IF concentrations in breast milk or cow's milk infant formulas are sufficient to exert significant hormonal effects. Estrogen concentrations in breast milk in the first few days of lactation (3 – 120 nM) are similar to IF concentrations, but decline thereafter [151]. In contrast, in children fed soy-based infant formulas IF plasma levels are 13 000 – 22 000 higher than the plasma concentrations of estradiol in early life (147 – 294 pM) whereas the contribution of IFs from breast milk or cow's milk is negligible. When values are expressed relative to body weight, the infant exclusively fed soy-based formulas is exposed to a dose that is 5 – 10-fold higher than the 0.7 mg/kg body weight *per* day intake shown to exert significant physiologic and beneficial effects on the hormonal regulation of women's menstrual cycle [152, 153].

4.3.3 IF intake by consumers of soy supplements

Extracted phytoestrogens are heavily marketed in numerous forms as dietary supplements. Consumers of these supplements are usually peri- and postmenopausal women looking for an alternative to hormone therapy. Although there are no approved health claims for phytoestrogens at this time, numerous claims are being made mainly regarding benefits to bone health and menopausal symptoms. The data sup-

Table 4. Isoflavone intake by infants

Reference	*N*	Age	Country	Infant food	Food consumption data	Mean IF intake (mg/kg bw/day)	Intake (mg/day)	Resulting mean plasma concentrations (nmol/L)
Setchell *et al.* [148]	7 *per* group	4 months	USA	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	4.5 – 8.0	28 – 47	3695 ± 1877
				Cow's milk formula				20.1 ± 1.2
				Human breast-milk				15.9 ± 3.0
Murphy *et al.* [110]	–	–	USA	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	5 – 12	n.g.	n.a.
Setchell *et al.* [149]		1 wk	USA	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	5.7 – 7.3	22.5 – 24.8	see Setchell *et al.* [148]
		1 month				6.0 – 11.9	31.5 – 36.0	
		2 months				6.1 – 10.0	36.0 – 37.0	
		4 months				6.0 – 9.3	41.0 – 45.0	
Irvine *et al.* [150]	4 *per* group	<1 month	New Zealand	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	3.0 ± 0.2	9.1 ± 0.7	n.a.
		1 month				3.8 ± 0.2	14.1 ± 0.6	
		2 months				3.3 ± 0.2	16.6 ± 1.1	
		4 months				2.9 ± 0.3	20.0 ± 2.0	
Friar and Walker [147]	–	1 – 2 months	UK	Soy-based infant formula	–	5	28	n.a.
		4 – 6 months				4.5	34	
Rupp *et al.* [143]	–	4 – 5 months	Switzerland	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	3.4 – 13.5	n.g.	n.a.
		>5 months				Max. 20		
Genovese and Lajolo [528]	–	0 – 2 wk	Brazil	Soy-based infant formula	Calculation according to analysis of soy-based infant formula, consumption, and bw	2.0 – 6.1	5.9 – 18.3	n.a.
		2 – 8 wk				1.7 – 6.6	6.6 – 26.2	
		2 – 3 months				1.6 – 5.2	8.2 – 26.2	
		3 – 6 months					9.2 – 34.9	
		>6 months				1.4 – 5.4	6.9 – 34.9	
Ryowon *et al.* [529]	9	10 months	Korea	Soy-based infant formula	Plasma analysis	n.g.	n.g.	1070.1 ± 68.0
		20 months						255.4 ± 10.7
Franke *et al.* [527]	11 *per* group	2 – 45 wk	USA	Human breast-milk	Plasma analysis	n.g.	n.g.	–
				Human breast-milk after mothers consume 55 mg IFs				19.7 ± 13.2 1049 ± 403

bw, body weight; n.a., not assessed; n.g. not given.

porting those claims are generally not strong (see Section 6).

Most supplements contain IFs derived from soybeans, red clover, or kudzu root and some contain botanicals such as black cohosh. In the case of soybean extracts, mainly the glucosylated IFs are present (see Section 2.1), whereas the red clover extracts mainly contain the IF aglycones [154]. Most product labels mention a content of about 40 – 50 mg *per* tablet. However, amounts of 100 and 200 mg can also be found [94, 113, 119, 155 – 157]. Analysis of the supplements has demonstrated that the actual IF content varied between products and was often less than declared on the label. Nurmi *et al*. [94] could show that only one product out of eleven was found to contain the same amount of IFs as was provided by the producer, while the other ten contained ±30 – 75% of the content as stated on the product label. Similar findings were reported by others [113, 154 – 158]. As glycosylation contributes considerably to the mass of IF molecules (about 40% in the case of the glucose unit and approximately 50% with respect of an acetyl- or malonlyglucose moiety), it is relevant for the producers to consider the total amount of potentially bioactive IF aglycones in supplements. Still, manufacturers usually neglect to state if contents are given in aglycone equivalents or in native forms [154]. Furthermore, the IF composition changes over time with total IF contents being constant [157]. Taken together, consumers cannot be sure that the declared IF content is correct.

5 Absorption, distribution, metabolism, and excretion (ADME) of IFs

The ADME as well as the bioavailability of phytoestrogens have not yet been fully elucidated. Most of the information is related to the ADME of daidzein, genistein and, to a lesser extent, glycitein.

5.1 Analytical methods to determine IFs in biological fluids

IFs occur mainly as glucuronide- and, to a lesser extent, sulfate conjugates in plasma and urine. For the determination of the total IF levels, the aglycones have to be liberated. In most cases, the required hydrolysis is performed enzymatically using Helix pomatia juice, an enzyme preparation containing β-glucuronidase and sulfatase activity. The hydrolysis step can be performed either after extraction of IF conjugates and metabolites from the centrifuged sample or directly after centrifugation of the sample. In the latter case, IF aglycones and metabolites are subsequently extracted from the hydrolyzed sample. Extraction is usually carried out with organic solvents, by SPE using C-18 RP cartridges or using a combination of both methods. In some cases, the extracts are purified by ion exchange chromatography or gel chromatography on Sephadex LH-20. IF aglycones are usually analyzed by GC-MS (after derivatization), HPLC with UV or coulometric electrode array detection as well as HPLC-MS. Nonchromatographic methods featuring high sample throughput and high sensitivity at comparatively low costs are immunoassays. Immunoassays are well suited for screening purposes but are often limited by cross reactivities with compounds which are structurally similar to the analyte. The individual approaches have been discussed in comprehensive reviews [120 – 126].

5.2 Absorption

One of the major questions has been whether the glycosidic forms of IFs must first be deconjugated to the respective aglycones by bacterial β-glucosidases or β-glucuronidases in the colon before absorption can occur or whether they can be transported across the small intestinal wall either intact or after deglycosylation by cytosolic or membrane-bound β-glucosidases. For flavonoids, *e.g*., quercetin or anthocyanins, the presence of small amounts of the intact glucosides in human plasma has been demonstrated [159, 160]. By studying the mechanisms of absorption evidence exists for the involvement of the lactase-phlorizin-hydrolase, and other intestinal β-glucosidases in hydrolysis of the glucosides and of the multidrug-resistance associated protein-2 (MRP-2) in the efflux of flavonoids [161 – 164].

Alternatively, the active transport of flavonoid glucosides by the sodium-dependent glucose cotransporter (SGLT1) and the subsequent cleavage of the glucosides by a cytosolic β-glucosidase has been discussed [161, 165]. Up to date there is little evidence that IF glucosides interact with SGLT1 [166, 167]. Moreover, a recent publication completely denied the transport of flavonoids *via* SGLT1 [168].

Relatively few studies addressed absorption of IFs. In a series of studies, the transport of IFs and their glycosides was examined using an isolated preparation of a luminally and vascularly perfused rat small intestine [169 – 171]. The aglycones were transported much more efficiently than their respective glycosides. In the case of genistein, only 1.3% of the glucoside passed the vascular side intact. The results are in line with *in vitro* studies using human intestinal Caco-2-cells [166, 167]. In contrast to the glycosides, the IF aglycones were able to penetrate the cells. Furthermore, IF glycosides were not detectable in human plasma after single dose administration to twelve women [172]. Taken together, these studies suggest that a large fraction of IFs is absorbed after hydrolysis to the respective aglycones.

5.3 Metabolism

5.3.1 Bacterial metabolism

Intestinal bacteria play an essential role in IF metabolism. For daidzein as well as genistein, the intestinal metabolism has been studied extensively *in vitro* and *in vivo* [173 – 180].

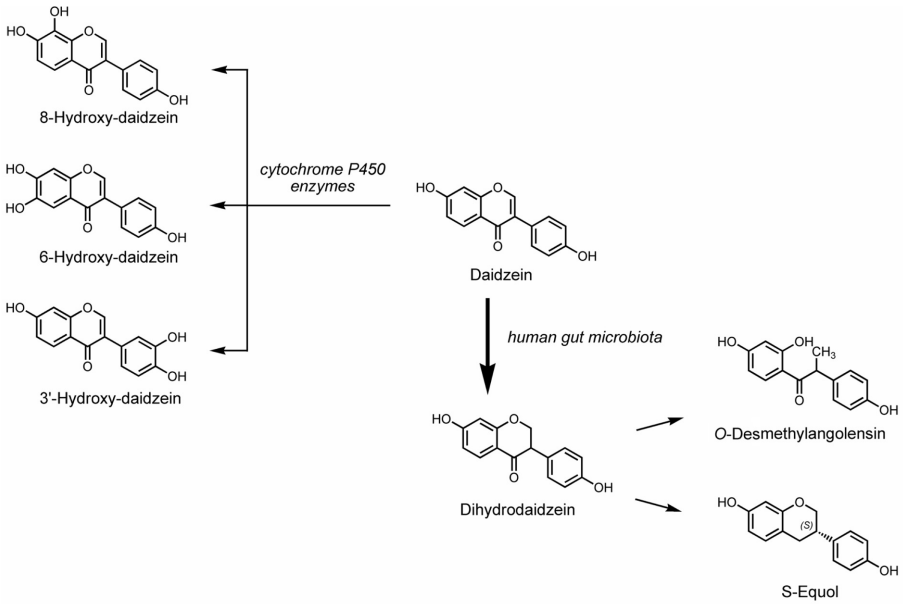


Figure 3. Metabolism of daidzein by the human gut microbiota (main metabolism route) and by cytochrome P 450 enzymes (minor metabolism route).

It has been reported that daidzein is converted by the gut microflora to the isoflavanone dihydrodaidzein (DHD), which can be further metabolized to both the isoflavane equol and the 5′-methyldeoxybenzoin *O*-desmethylangolensin (*O*-DMA) (see Fig. 3). Only 30% of the adult Western population and 50 – 60% of the Asians as well as vegetarians excrete equol in urine after having consumed soy foods – an observation which is still not understood [181]. Even when pure daidzein is administered which eliminates the influence of the food matrix, a high percentage of humans does not convert daidzein to equol. The equol producer is defined from urinary and serum equol concentrations. Unlike other IFs, equol has a chiral center and therefore can exist as two distinct optically active isomers, *R*- and *S*-equol, which are both bioavailable. Interestingly, the enantiomer produced by metabolic reduction from daidzein is known to be *S*-(–)-equol [182 – 184]. DHD also exhibits an asymmetric carbon atom, but its absolute naturally occurring configuration has not yet been determined.

Genistein is first – analogous to daidzein – reduced by gut bacteria to dihydrogenistein (DHG), followed by a cleavage of the C-ring to form 6′-hydroxy-*O*-DMA (6′-OH-*O*-DMA). The corresponding equol-derivative 5-OH-equol could not be identified yet. Instead, 6′-OH-*O*-DMA can be further degraded by the colonic microflora to yield 4-hydroxyphenyl-2-propionic acid. Decarboxylation can then lead to the putative metabolic end product 4-ethylphenol. Until now, 4-hydroxyphenyl-2-propionic acid has only been identified in rat urine and in *in vitro*-incubations with human microflora [178, 179]. The bacterial metabolism of genistein is depicted in Fig. 4.

Little is known about the bacterial metabolism of the third IF glycitein. Only recently, several reduced as well as reduced and demethylated metabolites of glycitein have been identified *in vitro* and *in vivo*, including 6-OH-daidzein, dihydroglycitein, 6-OH-DHD, 6-methoxy-equol, 6-OH-equol, 5′-OH-*O*-DMA, as well as 5′-methoxy-*O*-DMA [180, 185, 186]. It has not yet been assessed if the formation of 6-OH-equol is a general pathway or if only parts of the population are able to form this metabolite as in the case of the bacterial metabolism of daidzein. It should be pointed out that an alternative route for the formation of these metabolites is the biotransformation of daidzein (that means hydroxylation, reduction, and methylation). A definitive association between the parent compounds and the metabolites can only be made when tracer methods (radio or stable isotopes) or pure compounds are used [180].

Glycitein seems to be a rather stable molecule. The 4′-methyl ethers of daidzein and genistein – formononetin and biochanin A, respectively – are rapidly demethylated *in vitro* and *in vivo* which results in high plasma concentrations of daidzein and genistein. This is in contrast to glycitein, the methyl ether of 6-OH-daidzein [113, 186, 187].

Intestinal bacteria play an essential role in IF metabolism. Germ-free rats do not produce any of the above mentioned metabolites. After colonization of the rats with fecal microflora the metabolites are present in urine [188]. Furthermore, treatment with certain antibiotics causes marked reduction in bacterial metabolite production *in vitro* [189]. The appearance of these metabolites in plasma is time-dependent on their production in the colon. These metabolites appear in plasma several hours after IFs are consumed, presumably reflecting the time taken for unabsorbed IFs or IFs in the enterohepatic circulation, to reach the colon. A classical example of the appearance of bacterial metabolites in plasma is that peak equol concentration occurs at 24 – 36 h post ingestion of daidzein [190, 191].

Several candidate bacteria for the formation of these metabolites have been suggested, *e.g.*, *Escherichia coli* strain HGH21 reduced daidzein and genistein to the corre-

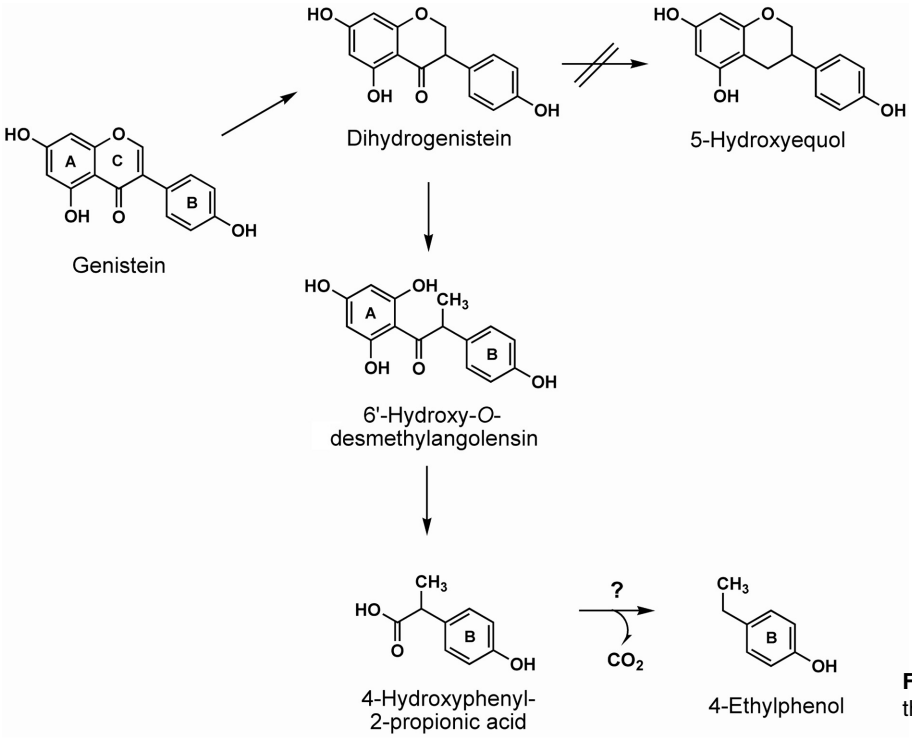


Figure 4. Metabolism of genistein by the human gut microbiota.

sponding dihydro-derivatives and a *Clostridium* sp., strain HGH136, cleaved the C-ring of daidzein to form *O*-DMA [192, 193]. Furthermore, *Eubacterium ramulus* is capable of producing *O*-DMA from daidzein and 6′-OH-*O*-DMA and 4-hydroxyphenyl-2-propionic acid from genistein as well as of cleaving the glycosidic bond from IF glycosides besides *E. coli*, strains HGH21 and 6 [194, 195]. *E. limosum* converts glycitein, formononetin, and biochanin A to the corresponding demethylated compounds 6-OH-daidzein, daidzein, and genistein [187]. The bacterium which produces equol from daidzein has recently been isolated from rat intestine [196]. It is a gram-positive rod-shaped bacterium *do03* (AB266102). Other bacteria identified in the human equol metabolism are *SNU-Julong 732* which converts DHD to equol as well as a mixture of *Lactobacillus mucosae* EPI2, *Enterococcus faecium* EPI1, *Finegoldia magna* EPI3, *Veillonella* sp. which is able to form equol from daidzein [197, 198].

5.3.2 Oxidative metabolism

Upon absorption IFs are metabolized by cytochrome P450 isoenzymes (P450) in the liver. Genistein and daidzein undergo hydroxylation catalyzed by P450 enzymes *in vitro* [199, 200]. Furthermore, several mono- and dihydroxylated daidzein and genistein metabolites – predominantly in the C-6, -8, and -3′ position – have been identified in human urine [180, 191, 200, 201] (see Fig. 3). P450 1A1, 1A2, 1B1, 2E1 as well as 3A4 have been identified to be involved in the phase-I-metabolism of genistein and daidzein [201].

Just recently, the oxidative metabolism of glycitein has been assessed. Several mono- and dihydroxylated metabolites as well as demethylated derivatives have been reported *in vitro* and *in vivo* [180, 185, 186]. The bacterial metabolite equol is also subject to phase-I-biotransformation *in vitro*. Ruefer *et al*. [202] identified 3′- and 8-OH-equol as main metabolites using human and rat liver microsomes.

5.3.3 Phase-II-metabolism

Once absorbed, IFs are efficiently conjugated, either with glucuronic acid or, to a lesser extent, sulfate. In addition, some sulfoglucuronides, diglucuronides, and disulfates may be formed. Conjugation takes place either in the liver or within the intestinal epithelium with UDP-glucuronosyl transferase or sulfotransferase enzymes ([203, 204], Ruefer, C. E. *et al*., Phase-II-metabolism of soy isoflavones and their metabolites *in vitro*, *J. Agric. Food Chem*., submitted). As a consequence, IFs are present in the circulation in predominantly conjugated forms. In human urine, 1 – 3% of genistein is found in the free form, 62 – 64% as monoglucuronide, 13 – 19% as diglucuronide, 6 – 12% as sulfoglucuronide, 2 – 3% as monosulfate, and 3 – 6% as disulfate [176]. The isoenzymes which efficiently conjugate IFs are UGT 1A1, 1A6, 1A8, 1A9, and 1A10 as well as SULT 1A1, 1A2, 1A3, 1B1, 1E1, 1C2, 2B1a, and 2B1b ([204, 205], Ruefer, C. E. *et al*., Phase-II-metabolism of soy isoflavones and their metabolites *in vitro*, *J. Agric. Food Chem*., submitted). Conjugation takes place predominantly at the hydroxyl group at C-7 [204 – 206].

5.4 Distribution

It has been shown that IFs and their metabolites are widely distributed within body fluids, but definitive tissue distribution studies have not been performed in human so far. Therefore, it is not known whether the measured plasma concentrations and the distribution of metabolites in the plasma are also representative for individual organs, particularly for potential target organs such as breast, prostate, and thyroid. Only a few data are available. IFs were detected in the breast tissue of premenopausal women and in the prostate fluid of men after the consumption of soy products. The concentrations of genistein and daidzein in the breast tissue were comparable to those found in plasma; however, the equol concentrations were higher [207, 208]. The prostate fluid was found to contain higher concentrations of IFs than plasma [209, 210]. In humans, the concentrations of unconjugated IFs in the plasma were relatively low. Data comparing the conjugated and unconjugated IF fraction in human tissue are not available. In rats, it was shown that tissues contain a much higher fraction of the aglycone compared to their plasma levels. For instance, the fraction of free genistein was 49% in the mammary gland tissue of the females, and even as much as 80 or 100% in the ovaries and the uterus, respectively, compared to less than 5% in the plasma.

5.5 Excretion

The main route of excretion of IFs is *via* the kidney. Fecal excretion appears to be minimal, but very few human studies have investigated this issue. Human trials have indicated that fecal excretion of daidzein and genistein lies within the range of 1 and 4% and urinary excretion between 5 and 35% of the ingested dose [211, 212].

5.6 Pharmacokinetics

IFs are readily absorbed from the gastrointestinal tract. In general, maximal plasma levels were achieved between 5 and 9 h after ingestion. Very often plasma concentration time curves exhibit two peaks: one early peak ~1–2 h after IF intake and the highest peak between 5 and 9 h. This biphasic shape is assumed to reflect the enterohepatic recycling as well as absorption occurring successively in the small intestine and in the colon. Elimination of IFs is quite slow, with half-life values of 6–8 h. A dose of 50 mg of either daidzein or genistein, yields a peak plasma concentration of ~2 μmol/L. IFs are therefore the most well-absorbed flavonoids [213]. Pharmaceutical doses of IFs lead to very high plasma levels: for example, doses of 4 and 8 mg genistein *per* kg body weight caused peak plasma concentrations up to 9.5 and 17.9 μmol/L, respectively [214]. The shape of the curve is not strongly modified by a long-term supplementation with IFs, neither for daidzein nor for genistein. Furthermore, the plasma concentrations do not return to baseline levels 24 h after IF intake [215].

5.7 Factors affecting the pharmacokinetics of IFs

There is a considerable interindividual variation in the pharmacokinetics of ingested phytoestrogens. For instance, a 12- and 15-fold interindividual variation for genistein and daidzein excretion, respectively, and a 600-fold interindividual variation of the equol excretion were reported after soy consumption [182, 216]. A variety of factors have been proposed to influence the pharmacokinetics of IFs. These include the composition of the intestinal microflora, the diet in general, the food matrix, food processing, chemical composition of IFs as well as age and gender.

5.7.1 Gut microflora

As pointed out in Section 5.3.1, the gut microbiota in general as well as the composition of the microbiota play an important role in the metabolism of IFs and thus has a substantial impact on the plasma concentration of IFs and their metabolites. Various physiological, pathological and environmental factors are likely to influence gut bacterial profile. These includes mainly the use of antibiotics and other drugs, bowel diseases as well as the diet, which has an impact on the gut motility, the intestinal transit time, the redox potential of the intestine, the gastro-intestinal pH as well as on the mucin and bile secretion. Also gender, genetics, and ethnicity may have a role in influencing the gut bacterial profile [217–219].

5.7.2 Diet, food matrix, and the chemical nature of IFs

The comparison of the pharmacokinetic data of various studies using different IF sources (*e.g.*, pure compounds, different formulated IF supplements, soy protein isolate, tofu, soymilk, tempeh) leads to the assumption that several factors, *e.g.*, the background diet, the food matrix, the degree of food processing as well as the chemical composition of the IFs directly influence their bioavailability and metabolism [220]. The data obtained in a single study are therefore usually the sum of several factors which might have an impact on the pharmacokinetics, a point which does not very often receive enough attention. Furthermore, it is very difficult to compare studies described in the literature retrospectively in detail, because necessary information on the associated food matrix and/or other food components ingested together with the IFs are often incomplete. If extracts or IF supplements were used, very often no information is given on the kind of pharmaceutical formulation, adjuvances, or matrix components, all important factors for drug release. This deficiency on important information can lead to confusing data. One prominent example is the discussion whether IFs are better absorbed in the glycoside or in the aglycone form. The data seem to be controversial:

there are reports that the glucosides are more bioavailable [113, 191, 221, 222] while others found no impact of the sugar conjugation [223, 224] or reported that IF aglycones were absorbed faster and in greater amounts [219, 225]. Yet, a more detailed look on the kind of IF preparations used in the studies may give in some cases explanatory approaches. For example, in one of the studies the bioavailability of an IF preparation called Fujiflavone P10 was investigated, where cyclodextrins were used as cathrate compounds for the IFs [223].

It seems doubtful that studies in which such special formulations have been used can contribute to the question whether glycosides or aglycones are better absorbed. In two further studies, pure IFs (nonformulated, no matrix components or coating) were consumed by the volunteers together with a drink and a following breakfast [113, 191, 221]. In both studies, the IF glycosides showed a higher bioavailability.

Only two studies were available in which different soy foods were used. In a recently published study, native soy milk (rich in glycosides), fermented soy milk (rich in aglycones), tempeh (mainly aglycones), and TVP (mainly glycosides) were compared. It was found that the aglycones were absorbed faster and in greater amounts than the glycosides [220] and that a liquid matrix such as soy milk yields a faster absorption rate and higher peak plasma concentrations than a solid matrix. Between the two solid foods, the IFs from tempeh (mainly aglycones) were more bioavailable than the IFs (mainly glycosides) in TVP. Beside the different chemical composition of the IFs, also the nutrient composition of tempeh and TVP is quite different, *e.g.*, in contrast to the nearly fat-free TVP, tempeh contains about 7–8% fat, which might affect the IF bioavailability.

5.7.3 Gender- and age-related influences on the bioavailability

Infants can absorb and excrete daidzein and genistein derived from soy-based infant formulas as efficiently as adults consuming soy products from the age of 4 wk on [150]. Also Setchell *et al*. [148, 149] reported that 4-month-old infants absorb IFs efficiently. The ability of infants to convert daidzein to *O*-DMA and equol during the first few months of life is limited. This has been attributed to the presence of an immature gut microflora. The gut is sterile at birth, but within a week of birth a microbiota begins to develop and the profile continues to change from infancy into adulthood. It is suggested that the ability to convert daidzein to *O*-DMA develops early in infants, while the ability to produce equol develops later [190]. Effects of age later on in life on the bioavailability and metabolism has not been reported so far [190, 220, 226].

Various studies reported that the concentrations of IFs and their metabolites in plasma and urine are sex-independent. In some other studies, differences for single pharmacokinetic parameters are shown. For example, data presented by Lu and Anderson [227] showed that during a 1-month trial in which soymilk was ingested the excretion half-life progressively shortened in women but lengthened in men throughout the trial. Wiseman *et al*. [228] reported significantly higher *O*-DMA plasma concentrations in men compared to women after consumption of soy products, whereas other parameters were not different. Cassidy [220] found gender differences in peak concentrations of daidzein, with higher levels attained in women.

6 Biological effects of soy IFs

6.1 Estrogen receptor (ER) mediated mechanism of action

IFs have a spatial configuration similar to that of mammalian estrogens, bind to ERs and affect estrogen-regulated gene products [229, 230]. The estrogenic potency of soy IFs is low compared to 17β-estradiol [231], with soy IFs having approximately 1/1000 and one-third of the affinity of 17β-estradiol for the ERα and ERβ, respectively [232]. The binding affinity of daidzein for ERα and ERβ is relatively low whereas genistein exhibits higher binding affinities [134, 232, 233]. Although the reported estrogenic potency of IFs [234] is weak compared with 17β-estradiol, their biological potential cannot be ignored, as typical circulating levels of IFs can manifold exceed endogenous estradiol concentration following consumption of a diet containing soy foods [134, 226].

The higher binding affinity of soy IFs for ERβ compared with ERα and the different tissue distributions of these receptors suggest these compounds may be tissue selective, exerting estrogenic actions in some tissues such as coronary vessels [235] but not in other tissues such as the endometrium [236–238]. Therefore, they are regarded as selective estrogen receptor modulators (SERM), substances, which have estrogenic effects in some tissues, but either no effects or antiestrogenic effects in others (for reviews on ERs and SERMs see [239–241]). Furthermore, it has been suggested that soy IFs may act as selective tissue estrogenic activity regulators (STEARs), compounds which provide estrogenic activity *via* other routes than direct interaction with the receptors or are precursors for *in vivo* metabolism to produce compounds with endocrine activity [242].

The formation of glucuronide conjugates decreases the relative affinities of IFs to ERs [243]. Genistein has a 100-fold greater binding affinity than daidzein for the mouse uterine cytosolic ERs. It needs to be taken into account that, binding affinity alone does not determine potency of the compound, because of the resulting conformational change in the ligand (IF-receptor complex varies among ligand regardless of binding affinity). The IF genistein is also >1000-fold more potent at triggering transcriptional activity with ERβ than ERα [244], and this difference is far greater than 20-fold greater binding affinity for ERβ than

ERα [245]. These data therefore suggest that the divergent transcriptional activities of estrogens and soy IFs result not only from their different binding affinities but also from differences in their ability to recruit coregulators and trigger transcriptional functions of ERα and ERβ.

6.2 Antioxidant activity in vitro

To date most of the investigations have focused solely on the antioxidant effects of genistein. Proposed molecular mechanisms responsible for its antioxidant potential include the ability to scavenge radicals, chelate metals, inhibit hydrogen peroxide (H_2O_2) production, and stimulate antioxidant enzymes, including catalase and superoxide dismutase.

In a liposomal system, genistein is a more effective antioxidant than daidzein, which is likely to be attributable to its third hydroxyl group in the C-5 position. Moreover, the IF precursors biochanin A and formononetin showed very weak antioxidant capacities in this *in vitro* system as they lack the hydroxyl group at the C-4′ position, which appears to be an important determinant of the antioxidant properties of IFs. Equol (daidzein metabolite; see Section 5.3.1) showed superior antioxidant actions [246] compared to both the precursor molecules and the parent IFs, suggesting that the absence of the 2,3-double bond in conjunction with a loss of the 4-oxo group enhances antioxidant properties [247]. Antioxidant activity, assessed by the trolox equivalent antioxidant capacity (TEAC) assay is consistent with these data that equol is a more potent IF compared with genistein and daidzein [248, 249].

In an *in vitro* experimental system, genistein (half maximal inhibitory concentration (IC_{50}) = 25 mM) was a more potent inhibitor of the formation of H_2O_2 by 12-*O*-tetradecanoylphorbol-13-acetate-activated HL-60 cells and the generation of superoxide anions by xanthine/xanthine oxidase compared with daidzein (IC_{50} = 150 mM), apigenin and biochanin A [250, 251]. Activities of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase were also significantly increased with genistein [250].

Genistein and daidzein undergo extensive metabolism in the gut and liver, which may affect their antioxidant properties. The antioxidant activity and free radical-scavenging properties of the IF metabolites equol, 8-OH-daidzein, *O-DMA*, and 1,3,5-trihydroxybenzene in comparison to their parent aglycones, genistein, and daidzein, have been investigated, with electron spin resonance spectroscopy. Results indicate that 8-hydroxy-daidzein was the most potent scavenger of hydroxyl and superoxide anion radicals. IF metabolites also exhibited higher antioxidant activity than parent compounds in standard antioxidant assays (ferric reducing ability of plasma (FRAP) and TEAC) indicating that the metabolism of IFs affects their antioxidant properties [252]. Furthermore, sulfation of IFs, which masks important hydroxyl groups of the IF molecule, could decrease their antioxidant activity and their impact on endothelial function [253].

IFs have been shown to reduce low-density lipoprotein (LDL) oxidation. Dietary supplementation with high-IF soy protein for 17 days prolonged the lag time of copper-induced LDL oxidation compared to a low-IF soy diet [254], confirming the results of an earlier smaller study [255]. In addition, the same study showed plasma concentrations of F2-isoprostanes (an *in vivo* marker of lipid peroxidation) were also reduced by the high-IF soy diet. *In vitro* data are consistent with these findings, with Kapiotis *et al*. [233] demonstrating that genistein inhibited the oxidation of LDL in the presence of copper ions or superoxide and NO radicals as measured by thiobarbituric acid-reactive substance (TBARS) formation.

6.3 Cardiovascular effects

Epidemiological studies suggest that differences in diet may explain the lower incidence of cardiovascular disease (CVD) in Japan compared with Western countries, and the wide international variability in intake of dietary IFs may play a role [97, 236, 256]. Several mechanisms of atheroprotective action have been proposed for soy IFs. Overall, their effectiveness in reducing the risk of CVD is not clear.

6.3.1 Blood lipids and atherosclerosis

6.3.1.1 Animal studies

The hypocholesterolemic effect of soy protein has been known for many decades. In many animal species, substituting soy protein for dietary animal protein consistently reduces LDL-cholesterol and total cholesterol levels [236, 257, 258]. Gerbils fed soy-based diets have significantly lower levels of total cholesterol, LDL + VLDL cholesterol, and of apolipoprotein B [236]. Feeding normolipidemic mice a synthetic cholesterol enriched diet with added soy protein isolate containing IFs led to a 30% decrease in plasma cholesterol levels and a 50% reduction in atherosclerotic lesion area compared to control animals fed the same cholesterol enriched diet but with added soy protein isolate without IFs. When the two diets were fed to LDL receptor deficient mice no differences in plasma cholesterol concentration and aortic atherosclerosis were recorded [259]. Addition of soy supplement with high IF content to a casein-based semisynthetic diet reduced aortic atherosclerosis in Watanabe heritable hyperlipidemic rabbits, a model of familial hypercholesterolemia, but had no effect on total cholesterol [260]. Soy protein containing IFs had no effect on total plasma cholesterol but decreased LDL-cholesterol and increased HDL-cholesterol in a group of female monkeys fed a moderately atherogenic diet [261]. When a state of menopause was experimentally established in this animal model, by ovariectomy, soy protein consumption, as com-

pared to casein consumption, significantly improved plasma lipids and lipoprotein concentrations [262]. Furthermore, feeding a diet with intact soy protein containing IFs reduced atherosclerotic lesions in ovariectomized, postmenopausal cynomolgus monkeys compared with controls given soy protein without the IFs [263]. Similarly, male cynomolgus monkeys fed an intact soy protein with IFs had significantly lower total and LDL plus VLDL cholesterol concentrations and the highest HDL cholesterol concentration compared to those fed soy protein with IFs mostly extracted or casein/lactoalbumin as a source of dietary protein. Also coronary artery atherosclerotic lesions were smallest in the group consuming an intact soy protein with IFs but the animals fed soy protein with IFs mostly extracted had also a significant reduction in coronary artery atherosclerosis compared to the animals receiving casein/lactoalbumin [264]. Feeding male cynomolgus monkeys atherogenic diets containing as a protein source either casein and albumin, or low IF soy protein (a mixture of unmodified protein isolate and IF depleted soy protein isolate), or unmodified soy protein isolate containing IF revealed beneficial effects on blood lipids (reduction of LDL cholesterol and increase in HDL cholesterol and apolipoprotein A) and on atherosclerosis (reduced mean plaque size in the coronary arteries) in both soy protein groups [265].

Thus the key issue of whether the response to soy protein is mediated through the presence of IFs has been the focus of much attention [6, 262, 266]. Most evidence relates to soy IFs but further studies are needed. Some animal studies suggested that soy protein with IFs may act atheroprotective by lowering plasma cholesterol by increasing LDL receptor activity with reduction of plasma cholesterol. However, other studies provide evidence for the LDL receptor- and plasma protein-independent pathways by which soy protein with IFs/soy IFs beneficially affect atherosclerosis in animal models. Furthermore, other bioactive, nonprotein components like the saponins might contribute to the cholesterol lowering effect of soy protein, a point which is very often not taken into account [267].

6.3.1.2 Human studies

Although the mechanism of action of the cholesterol lowering effect of soy is still poorly understood, soy has been used in the therapy of patients with hypercholesterolemia for several decades [268]. A 1995 meta-analysis of 38 clinical studies concluded that the mean reduction in serum total cholesterol was 9.3%, while LDL decreased by 12.9% with soy protein extracts [269]. Individuals with the highest initial cholesterol levels experienced the greatest reduction. Recent, more rigorous meta-analyses confirmed reduction of total and LDL-cholesterol and increase of HDL-cholesterol by soy protein both in adults with high or normal plasma cholesterol, but the overall effect was smaller than previously reported [269]. High-IF soy had a greater effect than low-IF soy, but little effect was found for isolated IFs alone [5, 270 – 273].

A few studies have presented evidence that IFs may play a role in lowering plasma LDL as their absence from soy may reduce the food's effectiveness in lowering cholesterol levels. A linear dose – response relationship was observed between dietary IF content and cholesterol reduction, with no lowering effect observed when IFs were removed from the soy protein in a group of 156 men and women with moderately elevated total cholesterol and LDL-cholesterol [274]. On the other hand, IFs alone fed as pure compounds or extract have been found to have no lipid-lowering effect [275] indicating that the mechanism of action of IFs on lipids is complex and probably involves an interaction with the food matrix. A recent evaluation of six reviews which have assessed the effects of soy IFs on lipid levels indicates that a diet supplemented with soy protein isolate containing IFs reduces LDL cholesterol by around 0.15 mmol/L, but without clear effect on HDL cholesterol or triglycerides. Furthermore, it indicates that the reduction in total cholesterol may be greater in men than in postmenopausal women, and that there is little evidence that the effectiveness of soy varies with baseline serum lipid levels, or the amount of IFs or soy protein consumed. None of the evaluated reviews suggests that purifed soy IFs or soy protein without IFs (where IFs were removed by alcohol wash) have statistically significant effects on blood lipids [276].

6.3.2 Arterial function

6.3.2.1 Possible mechanism of action

It has been hypothesized that the vasodilatory effects of soy IFs may be mediated through inhibition of tyrosine kinase, as a pharmacologically effective dose of genistein (a tyrosine kinase inhibitor) inhibited the contractile responses to noradrenaline in porcine coronary arteries [277] and in rat aorta without endothelium [278]. Supraphysiological genistein concentrations are required to inhibit tyrosine kinase activity [279], and other IFs, which are not tyrosine inhibitors, can potentiate nitric oxide production [280], suggesting that pathways other than tyrosine kinase inhibition are involved.

A series of experiments using aortic endothelial cells, explored the various possible mechanisms whereby acute physiological concentrations of genistein could induce endothelial nitric oxide synthase (eNOS) activation and demonstrated that inhibition of the PI3K/Akt and ERK/MAPK pathways did not affect genistein-induced eNOS activation [281]. An ER-antagonist also failed to abolish these effects, despite inhibiting the acute response to 17β-estradiol. The action of genistein as a tyrosine kinase inhibitor was excluded as the likely mechanism, since daidzein had weak but significant effects on eNOS activity (daidzein does not inhibit tyrosine kinase), plus 10 000-fold greater

threshold concentrations of genistein were required to inhibit tyrosine kinase activity in these cells. Finally, it was demonstrated that a protein kinase A (PKA) inhibitor completely abolished the genistein-induced eNOS activation. In addition, the PKA inhibitor did not inhibit eNOS activation by 17β-estradiol treatment, confirming the specific nature of this response. These results from bovine aortic endothelial cells were confirmed in human umbilical vein endothelial cells (HUVEC) [281]. Genistein was also shown to stimulate cyclic 3′,5′-adenosinemonophosphate (cAMP) production in the same experiments, confirming previous work with genistein showing the involvement of cAMP-dependent mechanisms in rat aortic rings [282]. Since PKA is cAMP-dependent, this suggests that genistein activates eNOS by stimulation of cAMP production leading to PKA-mediated phosphorylation of eNOS. These findings suggest an acute IF-specific pathway by which these estrogen-like components may activate eNOS, but need to be replicated by other groups, both *in vitro*, but more importantly *in vivo*. In contrast to Liu *et al*. [281], Joy *et al*. [283] demonstrated that genistein, daidzein, and equol stimulated NO production in HUVECs *via* ERK1/2- and PI3K/Akt- dependent eNOS phosphorylation pathways. In agreement with Liu *et al*. [281], it was shown that these effects were not affected by ER-antagonists [283]. The IF equol mediates rapid vascular relaxation.

On the one hand prostacyclin (PGI_2) expression is inhibited by supra-physiological concentrations of IFs in HUVECs [284]. On the other hand, serum from postmenopausal women who had taken IF supplements for 3 and 6 months induced a greater production of PGI_2 when incubated with HUVECs [285]. Furthermore, genistein and daidzein, in physiological doses, increased PGI_2 production in HUVECs *via* an ER-dependent mechanism, involving enhancement of cyclooxygenase-2 expression and activity [286]. A recent gene expression study provides further clues to the molecular mechanisms mediating the action of IFs on endothelial cells showing that genistein treatment in HUVECs modulated the expression of several key genes encoding for proteins involved in vascular tone, including endothelin converting enzyme 1, endothelin-2, estrogen related receptor-α, and atrial natriuretic peptide receptor A precursor [287].

6.3.2.2 *In vitro* and *in vivo* studies in normolipidemic and atherosclerotic animal models

The following studies evaluate arterial function as endothelium-mediated or endothelium-independent arterial dilation.

Genistein produced vasodilatation of rat mesenteric artery *in vitro* [288]. Genistein (at relatively high concentration >30 μM) potentiated endothelium-independent relaxation by isoproterenol in rat aortic rings [282]. Genistein but not daidzein increased relaxation induced by nitroglycerine a nitric oxide donor in rat aortic rings at concentrations of 10^{-5} M and above [289]. Genistein dose-dependently relaxed precontracted strips of rabbit aortic smooth muscle and this vasodilatory effect *in vitro* was endothelium independent and not related to the nitric oxide [290]. Other studies have shown increased endothelium-dependent relaxation of arterial rings following incubation with genistein and daidzein [291] and IF metabolites [292].

Genistein (0.2 mg/kg body weight, given subcutan for 4 wk) improved endothelium dependent relaxation in aorta and increased NO synthase (NOS) activity in lung homogenates in normocholesterolemic rats [293]. Acetylcholine-induced relaxation in aorta of normolipidemic ovariectomized rats slightly improved with a daily intake of 157 mg/kg soy IFs (containing 52% genistein, 42% daidzein, and 6% glycitein) in the diet for 1 month, whereas 67 mg/kg body weight had no effect [294]. Oral administration of soy extract improved endothelial dysfunction in ovariectomized rats [295], and reduced elevated blood pressure and endothelial dysfunction in spontaneously hypertensive rats. This effect seems to be related to increase endothelial nitric oxid synthase (eNOS) activity associated with increased calmodulin expression and decreased superoxide generation [296].

The effect of soy IFs on endothelium-dependent relaxation in atherosclerotic animal models is less clear. Synthetic IF metabolites (DHD and dehydroequol) demonstrated *ex vivo* vasodilatory effects on the abdominal aorta of apolipoprotein E-deficient mice [297]. Contrary, studies in primates do not show a clear relationship between consumption of soy/soy IFs and positive effects on arterial function. Although treatment with dietary soy IFs in atherosclerotic peripubertal female monkeys reversed coronary vasoconstriction to acetylcholine [298], another study in ovariectomized atherosclerotic monkeys showed no beneficial effect of soy IFs on coronary vasoconstriction, only when soy IFs were given in combination with 17β-estradiol [299]. Feeding an atherogenic diet with added IF-rich soy protein concentrate for 31 months had no effect on endothelium-dependent or -independent reactivity of coronary arteries in atherosclerotic adult male monkeys [265]. In ovariectomized homozygous Watanabe heritable hyperlipidemic rabbits, daily intake of a purified diet to which 2% of soy supplement containing 15% IFs had been added 16 wk had no effect on cerebral vascular reactivity, when the endothelium-dependent responses to carbamide chloride or N^{ω}-nitro-L-arginine methyl ester or the endothelium-independent response to sodium nitroprusside where measured [300].

6.3.2.3 Human studies

Habitual dietary IF intakes have been correlated with markers of endothelial function. A negative association between dietary IF intake and aortic stiffness was shown in 403 postmenopausal Dutch women [301]. It should be considered however, that not all cross-sectional studies have shown the same [302].

Table 5. Human intervention trials: effects of IFs on in vivo endothelial function and putative biomarkers of endothelial function

Reference	Subject characteristics	Study design	Duration of study	Food/supplement description	Dose aglycone composition (mg/day)	Measure	Outcome
Atkinson *et al*. [530]	177 pre-, peri-, and postmenopausal W.	R SB P Pa	12 months	43.5 mg/day red clover IF tablets.	26 mg/day biochanin A, 16 mg/day formononetin, 1 mg/day genistein, 0.5 mg/day daidzein	Fibrinogen PAI-1	NC ↓ (perimenopausal only)
Colacurci *et al*. [531]	57 PMW Italy	R DB P Pa	6 months	120 mg/day soy IF tablets *versus* placebo	60 mg/day genistein, 60 mg/day daidzein	FMD VCAM-1 ICAM-1 E-selectin P-selectin Thrombomodulin vWF TPA	↑ ↓ ↓ ↓ ↓ NC NC NC
Hale *et al*. [532]	29 PMW >1 year since last menstruation USA	R DB P Pa	2 wk	80 mg/day soy IF tablets *versus* placebo	Not reported	FMD	NC
Hall *et al*. [324]	117 PMW >1 year since last menstruation UK, Italy, Germany, and Denmark	R DB P CO	8 wk *per* arm	50 mg/day soy IF-enriched cereal bars *versus* placebo cereal bars with no IFs added	~33 mg/day genistein, ~17 mg/day daidzein	VCAM-1, ICAM-1, E-selectin, MCP-1, vWF, ET-1	NC
Hallund *et al*. [533]	30 PMW >1 year since last menstruation Denmark	R DB P CO	8 wk *per* arm	50 mg/day soy IF-enriched cereal bars *versus* placebo cereal bars with no IFs added	~33 mg/day genistein, ~17 mg/day daidzein	FMD Endothelium-independent vasodilation Arterial Compliance NOx	NC ↑ ↑ (after placebo) ↑
Lissin *et al*. [534]	40 PMW USA	R	6 wk	90 mg/day soy IF tablets	44.6 mg/day genistein, 44.6 mg/day daidzein, 0.8 mg/day glycitein	FMD Endothelium-independent vasodilation	NC ↑
Nestel *et al*. [275]	28 Perimenopausal and PMW Australia	R SB P CO	15 wk *per* arm	80 mg/day soy IF tablets *versus* placebo	45 mg/day genistein, 33 mg/day daidzein, 2 mg/day glycitein	Arterial compliance	↑
Nestel *et al*. [535]	14 PMW >1 year since last menstruation Australia	R DB P sequential treatments	15 wk (5 wk treatment)	40 or 80 mg/day red clover IF tablets, *versus* placebo	40 mg tablet: 4 mg genistein, 3.5 mg daidzein, 24.5 mg biochanin A, 8 mg formononetin	Arterial compliance	↑
Nikander *et al*. [536]	56 PMW History of breast cancer. Finland	R DB P CO	3 months	114 mg/day IF tablets	66 mg/day glycitein, 42 mg/day daidzein, 6 mg/day genistein	E-selectin NOx	NC NC
Simons *et al*. [537]	20 PMW >1 year since last menstruation Australia	R DB P CO	8 wk *per* arm	80 mg/day soy IF tablets *versus* placebo		FMD	NC
Squadrito *et al*. [538]	60 PMW >1 year since last menstruation Italy	R DB P Pa	6 months	54 mg/day genistein tablets (n 30) *versus* placebo	54 mg/day genistein	NOx Endothelin-1 FMD	↑ ↓ ↑

Table 5. Continued

Reference	Subject characteristics	Study design	Duration of study	Food/supplement description	Dose aglycone composition (mg/day)	Measure	Outcome
Squadrito *et al.* [539]	79 PMW >1 year since last menstruation Italy	R DB P Pa	12 months	54 mg/day genistein tablets (n 27) *versus* HRT or placebo	54 mg/day genistein	NOx Endothelin-1 FMD	↑ ↓ ↑
Teede *et al.* [540]	80 M and W Australia	R DB P CO	6 wk *per* arm	80 mg/day red clover IF tablets *versus* placebo	80 mg/day biochanin A (n 40) or formononetin (n 40) *versus* placebo	Arterial stiffness VCAM-1 FMD	↓ (formononetin) ↓ (formononetin) NC

R, randomized; SB, single-blind; DB, double-blind; P, placebo controlled; Pa, parallel; CO, crossover design; M, men; W, women; PMW, postmenopausal women; NC, no change; PAI-1, plasminogen activator inhibitor-1; FMD, flow-mediated dilatation; NOx, nitric oxide metabolites; HRT, hormone replacement therapy; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willebrand Factor; MCP-1 monocyte chemoattractant protein; TPA, tissue plasminogen activator antigen.

A number of human intervention studies have investigated the effects of isolated soy IF supplementation on endothelial function (Table 5). A number of studies demonstrated positive effects on *in vivo* endothelial function whereas other studies found no effect.

Since biomarkers of endothelial function differ between studies, it is not yet possible to draw conclusions regarding effects of soy IFs on endothelial function. Some of the human intervention studies have used small subject numbers and circulating biomarkers may be more useful for large subject populations as an indicator of endothelial function. Overall, whilst these studies provide some promising indicators, there is need for replication of the positive findings, in other laboratories and using a wider range of direct measures of endothelial function.

6.3.3 Inflammation, cell adhesion, and platelet aggregation

The potential anti-inflammatory and cell adhesion properties of IFs have been tested in several *in vitro* model systems. Importantly, to date most of the studies conducted have used concentrations of IFs that are unlikely to be achievable at physiologically relevant concentrations. Activation of the endothelium results in the release of vascular cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α). These cytokines in turn induce the leucocyte adhesion molecule E-selectin, the cell surface expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), which are centrally involved in the endothelial recruitment of leucocytes [303]. Focal expression of ICAM-1 and VCAM-1 in arterial endothelium overlying early foam cell lesions together with the activation of monocyte chemoattractant protein-1 (MCP-1), leads to infiltration of mononuclear cells into the artery wall [304]. The uptake of oxidized LDL by these cells leads to the formation of lipid-laden foam cells, and the development or progression of atherosclerotic plaques [304].

Transcription of ICAM-1, VCAM-1, and MCP-1 is dependent, at least in part, on the activation of the transcription factor, NF-κB.

At pharmacological concentrations, genistein (40 μM) attenuated both NF-κB DNA binding and TNF-α release in human monocytes [305]. Furthermore, soy-IF supplementation for 3 wk reduced *ex vivo* NF-κB activation induced by TNF-α in the peripheral lymphocytes of healthy men [306].

To date several studies have investigated the potential intracellular signaling mechanisms of genistein, which would result from expression of cytokines IL-1 and TNF-α. Genistein dose-dependently inhibited TNF-α- and IL-1-stimulated E-selectin expression and VCAM-1 secretion in HUVEC, although ICAM-1 was unaffected [307]. Furthermore, pretreatment of HUVEC with genistein resulted in a dose-dependent inhibition of TNF-α-induced E-selectin, VCAM-1, and ICAM-1 surface expression [308].

Monocyte-derived macrophages are the principal inflammatory cells in the atheromata. In early stages of artherosclerotic lesion formation, macrophages and endothelial cells interact to trigger a cycle of events that exacerbates endothelial dysfunction, resulting in a loss of homeostatic control [309]. Activated macrophages generate large amounts of nitric oxide (NO) from L-arginine by the action of inducible NO synthase (iNOS) and its overproduction has been associated with oxidative stress and chronic inflammation [310]. Genistein inhibited nitrite production in a dose-dependent manner when rat mesangial cells were activated with IL-1β, suggesting a central role for protein tyrosine kinase in the signaling pathway of IL-1β, resulting in the activation of iNOS in rat mesangial cells [311]. In a further study, genistein (IC_{50} = 58 μM) and daidzein (IC_{50} = 107 μM) inhibited NO production in activated macrophages [312]. In

that study, iNOS mRNA levels remained unchanged by the IF treatment, which suggests that the inhibitory effect is post-transcriptional. This suppression of lipoylsaccharide (LPS) activity has also been shown for other plant phenolics [313].

MCP-1 may play a key role in atherogenesis since it is involved in the recruitment of monocytes and T-cells into the arterial wall. MCP-1 mRNA has been detected in atherosclerotic lesions by *in situ* hybridization [314]. Furthermore, a decrease in atherosclerotic lesion size is seen in mice deficient in the MCP-1 receptor CCR-2, and fewer macrophages and monocytes are present in their aortas [315]. Recently, it has been shown that genistein (IC_{50} = 29 μM) and daidzein (IC_{50} = 37 μM) dose-dependently down-regulated MCP-1 secretion [312], indicating that both of these IFs may have the potential to inhibit monocyte infiltration into the arterial wall. It is known that the expression of MCP-1 is regulated at the transcriptional level [316]. Therefore, genistein and daidzein may regulate TNF-α-induced MCP-1 expression through transcription factors such as NF-κB.

Both genistein and daidzein exert antiaggregatory activity in human platelets *in vitro* [312]. This finding is consistent with earlier reports that the consumption of soy protein and its IF-enriched fraction lowers platelet aggregation in rats [317].

The exact molecular mechanisms by which IFs affect platelet aggregation are unclear and are currently under investigation. The modification of platelet cAMP *via* the inhibition of phosphodiesterase activity is the most supported pathway for antiaggregatory effects of flavonoids [318]. Inhibition of lipoxygenase activity, as demonstrated principally for myricetin and quercetin [319], is another possible mechanism. Stimulation of adenylate cyclase, leading to increased cAMP levels, has been proposed as a further antiaggregatory signal transduction pathway [320]. Platelet aggregation was shown to be associated with the production of H_2O_2, which acts as an important second messenger in platelets [321]. Furthermore, platelets primed with nonactivating concentrations of arachidonic acid or collagen were activated by nanomolar concentrations of H_2O_2 [322]. Since IFs may scavenge free radicals, this evidence that reactive oxygen species are involved in platelet stimulation suggests another antiaggregatory mechanism.

6.3.4 Genetic polymorphisms as a determinant of biological response to soy IFs

The effects of IFs on cardiovascular risk may vary between individuals. As well as interindividual variation due to equol production status, genetic variation may be an important determinant of biological responses to IF consumption. An increasing number of studies are being published that demonstrate the significant impact of genetic variation on changes in cardiovascular risk factors in response to dietary intervention [323]. This has recently been reported for dietary IFs and genetic polymorphisms, and may help to explain some of the disparities in the current literature on IFs and cardiovascular health.

IF – genotype interactions are a novel, and therefore poorly understood area of nutrition. Investigation of the influence of apoE genotype on IFs and cardiovascular risk factors in postmenopausal women showed no significant IF – genotype interactions, but these data were hampered by small sample size in the rarer genotype groups [324].

Polymorphisms in the ER are prime candidates for potential IF – genotype interactions, since this is a highly plausible mechanism by which response to IFs may differ between individuals. Existing evidence that single nucleotide polymorphisms (SNPs) in the ERα-gene affect HDL cholesterol and E-selectin response to hormone replacement therapy is supportive of this hypothesis [325, 326]. IF – genotype interactions were revealed for 2 SNPs in the ERβ-gene, but no biomarker associations were found between response to IFs and ERα-genotypes in postmenopausal women [324, 327]. In these women, a reduction in plasma VCAM-1 following IFs consumption compared to placebo was recorded in the variant AA genotype but not the GG or GA genotypes of the ERβ-*Alu*I polymorphism [324]. This agrees with previous mechanistic studies that show estrogen and genistein inhibit VCAM-1 expression in cultured endothelial cells *via* ER-mediated mechanisms [328, 329]. Furthermore, there is evidence that cardiovascular risk varies according to the ERβ-*Alu*I polymorphism. A cross-sectional study demonstrated that there was a higher frequency of the AA genotype in patients with premature coronary artery disease (CAD) compared to healthy controls. In addition, there were increased mean plasma triglyceride and apo B concentrations, decreased plasma HDL cholesterol concentrations and increased BMI in the AA genotypes of healthy and CAD patients compared to the GA and GG genotypes for the ERβ-*Alu*I polymorphism [330]. Therefore, it appears that the ERβ-*Alu*I AA genotype is associated with an increased cardiovascular risk, but it is this group that benefits from dietary IFs (in respect to plasma soluble VCAM-1) to a greater extent than the other genotypes.

In summary, IF-rich soy foods, soy protein isolate, and IF extracts have a variety of effects that may influence cardiovascular risk. The effects of soy protein on blood lipids appear to be related to components other than IFs, with specific subunits of 7S soy globulins suggested to be responsible [331], although there may be an interaction of IFs with the soy matrix that influences the efficacy of lipid-lowering effects. Possibly, other mechanisms that mediate the development of atherosclerosis may be influenced by dietary IF intake, such as LDL oxidation, vascular inflammation, platelet aggregration, and endothelium-dependent vasodilation. Finally, individual response to dietary IFs may depend on the ER genotype with regard to biomarkers of cardiovascular risk.

6.4 Effect on cancer

6.4.1 Breast cancer

6.4.1.1 *In vitro* data

The effects of soy IFs on the proliferation of breast cancer cells *in vitro* have been well documented. At low, physiologically relevant concentrations (~1 μM), genistein, daidzein, and equol stimulate the proliferation of the ER positive (ER+) MCF-7 breast cancer cell-line [332–337]. In contrast, at higher concentrations (>10 μM), genistein is a potent inhibitor of both ER+ and ER– breast cancer cells [332–336]. The stimulatory effects induced by genistein and equol have been shown to correlate closely with their binding affinities for the ER [336] suggesting that at low concentrations, IFs act *via* an ER-dependent mechanism. At high concentrations genistein has been shown to induce G2/M cell cycle arrest in both ER+ and ER– breast cancer cells [338–343] which is associated with an increase in p21WAF1/CIP1 expression and subsequently apoptosis [338, 339, 342–344]. These effects occur irrespectively of ER and p53 status [342, 344], thus implicating estrogen-independent mechanisms of action.

Soy IFs have also been shown to play an important role in the process of tumor invasion *in vitro*. Valachovicova *et al*. [345] have shown that the aglycones genistein, daidzein, and glycitein and their respective glycosides markedly reduce the motility of highly invasive MDA-MB-231 breast cancer cells, probably *via* inhibition of the transcription factors NF-κB and AP-1. Furthermore, using the matrigel invasion assay, genistein, daidzein, glycitein, and equol have been shown to inhibit the invasion of MDA-MB-231 breast cancer cells [346–349], though significant effects are only apparent at relatively high IF concentrations (10–75 μM). The mechanism appears to be by down-regulation of matrix metalloproteinases 2 and 9 and up-regulation of tissue inhibitor TIMP-1 [346, 347, 350].

Other possible mechanisms of action of the soy IFs in breast cancer include inhibition of angiogenesis [351, 352], inhibition of enzymes involved in estrogen biosynthesis and metabolism [334] and antioxidant properties [353]. Genistein has also been shown to up-regulate the phase II detoxification enzymes quinone reductase in breast cancer cells [354] and glutathione *S*-transferase in nonneoplastic mammary cells [355], thus conferring protection against genotoxic effects.

The findings that low, physiologically relevant doses of IFs stimulate breast cancer growth *in vitro* has raised concerns, especially among women at high risk of breast cancer. It should be noted, that breast tissue IF concentrations have been found to be 2–3 × higher than paired serum levels [356], suggesting that breast tissue may be exposed to much higher IF concentrations than initially thought. An issue of concern, are the adverse effects that IFs have been shown to exert on tamoxifen action in breast cancer cells. Jones *et al*. [357] have shown that 1 μM genistein reversed the inhibitory effects of tamoxifen (G1 arrest and inhibition of proliferation) in T47-D breast cancer cells. Similar results were obtained in tamoxifen-sensitive MCF-7 breast cancer cells [358]. In contrast, Zava and Duwe [336] found that tamoxifen sensitized T47-D breast cancer cells to genistein-induced growth inhibition (2–10 μM). Furthermore, genistein and tamoxifen have recently been shown to synergistically inhibit the growth of ER+/HER2-overexpressing BT-474 breast cancer cells [359]. These conflicting results warrant further investigation in order to elucidate the potential role of genistein in combination with chemotherapeutic agents in the treatment of breast cancer.

The *in vitro* biological effects of the daidzein metabolite equol have received much attention recently following the suggestion that the interindividual variation in the ability to metabolize equol from daidzein, a conversion that only 30–40% of adults in low soy-consuming populations can perform [182, 360], may have implications for disease risk [361]. *In vitro* studies have demonstrated that equol has a higher affinity for the ER than daidzein [362, 363], is 100-fold more potent than daidzein in stimulating pS2 expression in MCF-7 breast cancer cells [363] and has stronger antioxidant activity compared with other IFs [246, 247, 249, 364].

In conclusion, it is evident from *in vitro* studies that soy may mediate biological effects *via* a variety of mechanisms and may confer protection against breast cancer development at all stages of the carcinogenic process depending on the dose used.

6.4.1.2 Animal studies

There are conflicting data concerning the effect of soy IFs on breast cancer. Both cancer protective and cancer promoting effects on tumor development have been reported in animal studies. The growth of estrogen-dependant human breast cancer (MCF-7) tumors implanted in athymic mice has been found to be stimulated by genistein and daidzein [332, 365, 366]. In the same animal model and in a transgenic animal model, it has further been observed that both IFs can negate the effect of tamoxifen [367, 368]. In a breast cancer model based on Sprague–Dawley rats daidzein in combination with tamoxifen was found to be more effective in cancer prevention than tamoxifen alone, whereas a combination of genistein and tamoxifen had the opposite effect [369]. A study done in transgenic mice did not detect any interaction of soy IFs and tamoxifen [370] indicating that more research is needed before any conclusion can be made. Several studies using both transgenic animals as well as the 1-methyl-1-nitrosourea (MNU) induced mammary carcinogenesis model in Sprague–Dawleys rats indicate that soy IFs can stimulate breast tumor growth [371–375]. Although there is some evidence that IFs can promote breast cancer several animal studies observed a cancer protective effect of soy IFs [7, 370, 376–394]. The time of exposure seems to be critical for the effect of soy IFs on

breast cancer. Several studies by Hilakivi-Clarke *et al.* [395] and Lamartiniere and coworkers [386, 391, 395, 396] indicate that prepubertal exposure to genistein and daidzein results in a cancer protective effect, whereas *in utero* and adult exposure was not found to protect against breast cancer in rodents. On the contrary, *in utero* exposure to genistein was observed to promote breast cancer development [396 – 398]. In the majority of studies observing a protective effect, *i.e.*, increased latency period, decreased tumor multiplicity and suppression of tumor promotion, animals were exposed both throughout the prepubertal and the adult period [376, 378, 381, 386, 388 – 392]. Studies not observing an effect on breast cancer were those where adults were exposed to soy IFs [378, 399 – 401]. Genistein administered to animals after tumor development has been observed to reduce metastasis and tumor induced angiogenesis [380, 393]. Another factor that has been detected to be important for the effect on mammary carcinogenesis in animal studies is the background diet and the degree of soy processing [383, 402, 403]. Adding soy IFs to a Western style diet does not protect against mammary tumorigenesis; on the contrary, it was observed to reduce tumor latency [403]. On the other hand, supplementation with IFs of a rodent chow diet based on soy protein was found to reduce mammary tumorigenesis whereas soy IFs added to a purified diet did not affect breast cancer development in the same study [383].

In conclusion, animal studies provide evidence that time of exposure is crucial for the effect of IFs on breast cancer development and there is some evidence that the choice of diet could influence the outcome of animal studies investigating the effect of soy IFs on breast cancer. Although several animal studies confirmed cancer preventing effects of a soy-based diet [384, 404 – 406], other investigations in laboratory animals dosed with extracts containing soy IF demonstrate increased incidence of mammary tumors, or indicate that IFs can promote breast cancer [371, 374, 398, 402, 403, 407]. Therefore, the increased use of IF rich supplements by Western women should be regarded with caution. The Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG) has recently published a statement [408] focused on this issue (DFG Senate Commission on Food Safety). The reported carcinogenic effect of IFs in laboratory animals should be further elucidated.

6.4.1.3 Human data

The etiology of breast cancer is still poorly understood. Only 5 – 10% of all new diagnosed breast cancer cases are attributed to inherited factors [409]. International cancer statistics indicate that Asian populations have low rates of breast cancer, while Western populations have cancer rates that are many times higher [410 – 412]. For example, the current mortality rate for premenopausal breast cancer is approximately four-fold higher in the Western World than in the Far East Asian nations [413]. Epidemiological studies of breast cancer among Asian emigrants to the US show that the second generation, but not the first, acquire a breast cancer risk comparable to the host nation [224, 414]. This may indicate a role of environmental factors. One major environmental factor thought to influence the breast cancer incidence is nutrition. An obvious difference between the traditional Asian and Western diet is the higher intake of plant-based foods, especially soy and soy-derived foods in the Asian diet. This results in higher intake of IFs, one group of potentially natural chemoprotective compounds in soy [404, 415]. A recent meta-analysis of 18 epidemiological studies (12 case control and 6 cohort or nested case control) reveals a small inverse association between soy intake and breast cancer intake in both Western and Asian women [416]. Noteworthy, the authors stated that this result should be interpreted with caution due to the potential exposure misclassification, confounding, and lack of a dose response [416]. Further studies are required to address the potential effect of soy and soy IFs on breast cancer and to address the current concerns for breast cancer survivors.

6.4.2 Prostate cancer

6.4.2.1 *In vitro* data

Many *in vitro* studies have been carried out using high IF concentrations (up to 100 μM), that is much higher than plasma IF levels (around 1 – 6 μM) achieved through consumption of a soy-rich diet [210, 417, 418]. It is interesting to note that there is evidence that IFs are concentrated in prostatic fluid at concentrations up to 47 μM [209, 210, 419].

A variety of studies have demonstrated that IFs can inhibit growth of both human prostate cancer cell lines, and cell lines derived from normal human prostatic epithelium [420 – 425]. These growth-inhibitory effects appear to be exerted *via* modulation of the cell cycle, although the stage of the cycle affected seems to vary with concentration and type of IF. For example, Hedlund *et al*. [422] found that while genistein (100 μM) arrested growth of a benign human prostatic epithelial cell line (PrEC) in G2/M, equol and daidzein (both at 100 μM) caused growth arrest at G0/G1. A wide range of cell cycle regulatory proteins are modulated by genistein in prostate cancer cells including cyclin B cyclin D1 and cyclin E, p21(waf1), p27, p53, CDK4, CDK2 [421, 426 – 430].

A common effect on androgen responsive genes, and genes associated with the IGF-1 pathway and MAP kinase related pathway was reported in a DNA microarray analysis on LNCaP cells treated with genistein, daidzein or equol (1 – 25 μM) [431, 432].

A further proposed mechanism of growth inhibition by genistein in androgen-independent DU145 cells is inhibition of telomerase activity by the transcriptional down-regulation of human telomerase reverse transcriptase (hTERT) *via* c-Myc and also posttranslational modification of hTERT pro-

tein *via* the deactivation of Akt [433, 434]. In addition, a recent paper has implicated genistein at physiological concentrations (0.5 – 1 μM) in the induction of BRCA-1 and BRCA-2 genes in DU-145 and LNCaP cells [435].

Several studies have reported that growth inhibition by IFs is associated with apoptosis [426, 428, 429], possibly *via* inhibition of the NF-κB pathway. Genistein has additionally been shown to induce the expression and activation of caspase-3, a protease involved in apoptosis, in LNCaP and DU145 cells [436].

The age-related increase of prostate cancer is associated with a progressive accumulation of oxidative DNA damage which is presumably associated with a decline of the cellular antioxidative defense during aging. Protection against hydrogen peroxide induced DNA damage by genistein (1 – 30 μM) in LAPC-4 cells was reported [437]. The protective effect was associated with induction of three genes encoding products with antioxidant activities, namely glutathione reductase, microsomal glutathione *S*-transferase 1 and metallothionein 1X.

Androgens are essential for the normal growth and physiological functioning of the prostate, and also play a role in the development of prostate cancer [438, 439]. Genistein at low, physiological concentrations (0.1 – 1 μM) down-regulated the androgen receptor at both mRNA and protein level in LNCaP cells, possibly *via* ER-β [440]. Down-regulation of expression of prostate specific antigen (PSA; a biomarker used in the diagnosis of prostate cancer) by genistein in LNCaP cells has been demonstrated in a number of studies [420, 424, 440, 441] but the concentrations of genistein may be critical. Gao *et al*. [442] report that low genistein concentrations (0.1 – 10 μM) activated androgen receptor-driven gene expression, while higher concentrations (100 μM) were inhibitory.

A number of *in vitro* studies have demonstrated inhibition of invasion and metastasis by IFs. For example, invasion of both PC3 and PC3-M cells through a Matrigel matrix is inhibited by 10 – 30 μM genistein [443, 444].

Angiogenesis is a critical process in the growth of tumors and vascular endothelial factor (VEGF) is thought to be an important regulator of the process. Studies by Guo *et al*. [445] indicate that genistein may inhibit angiogenesis in prostate tumors by suppression of VEGF mediated signaling pathways between the tumor cells and the vascular endothelium.

In conclusion, although many *in vitro* studies have been carried out using high, nonphysiological IF concentrations, there is considerable evidence to suggest that isoflavonoids can modulate a wide range of signaling cascades controlling prostate cell growth, proliferation, invasion, and angiogenesis.

6.4.2.2 Animal studies

In general, genistein and soy are found to protect against prostate cancer in animal studies. Dietary genistein and soy IF mixtures were detected to suppress prostate cancer both in animal models based on transgenic animals [446] and in chemically induced prostate cancer models [385, 446 – 450]. Genistein was furthermore observed to protect against chemically induced prostate cancer in a dose-dependent manner [385]. Besides the effect on prostate cancer development soy IFs and genistein were detected to inhibit the growth of human prostate cancer xenografts transplanted in rodents by inducing apoptosis in tumor cells and due to anti-angiogenesis [392, 451 – 456]. Several studies detected reduced serum testosterone levels following exposure to soy IFs [454, 456], which could explain the preventing effect on androgen dependent prostate cancer. Genistein was additionally observed to enhance prostate cancer treatment, to inhibit metastasis and to improve survival [392, 457 – 459]. Exposure to soy also was detected to inhibit prostate tumor growth and metastasis [278, 451, 452, 454 – 456]. However, it has been suggested that the molecular pathways of the prostate cancer protective effect is different for dietary soy, soy IFs and genistein [456], but these data need further investigations.

In conclusion, animal prostate cancer studies provide evidence that genistein as well as soy protect against prostate cancer and that soy IFs have the potential to inhibit tumor growth and metastasis.

6.4.2.3 Human data

6.4.2.3.1 Epidemiological evidence

Migrant studies suggest that external factors, such as diet, play a strong role in the development of prostate cancer. For example, while the incidence of prostate cancer in Japan is low, incidence among men of Japanese ancestry living in Hawaii is around ten times greater, although still below that of Hawaiian natives [460]. Other migrant studies indicate a similar pattern of changes in incidence for Asian immigrants into the USA [461 – 463]. It is noteworthy that levels of IFs and their metabolites are significantly higher in the plasma/serum, urine, and prostatic fluid of Asian men than in biological fluids of Western or European men [134, 135, 209].

Several case-control studies have also implicated soy consumption in prostate cancer prevention. A multicenter case-control study of African-American, White, Japanese, and Chinese men found that intake of soy foods was inversely proportional to prostate cancer risk [464] and a study in Caucasians reported greater consumption of foods containing genistein and daidzein in controls *versus* prostate cancer cases [465]. Similar findings have been reported in Chinese and Japanese case-control studies [466 – 468]. In contrast, a Canadian study (1623 cases and 1623 controls) [469] did not detect any relationship between tofu or soyfood intake and prostate cancer risk.

Prospective studies are less conclusive – of three studies, two, in men of Japanese origin, showed no significant relationship between soy product consumption and cancer risk

[460, 470] whereas a third study in 12 395 California Seventh-Day Adventist men [471] revealed a significant, 70% reduction in prostate cancer risk among those men consuming soy milk more than once a day.

6.4.2.3.2 Human intervention studies

Most human intervention studies have measured changes in serum prostate PSA as a way of assessing the impact of soy products or IF supplements on prostate cancer risk. It is expressed primarily by cells of the prostatic epithelium and while usually present at negligible levels in the blood, both cancer of the prostate and benign prostatic hyperplasia can cause blood PSA levels to rise dramatically. The results of these studies are highly variable, possibly reflecting the heterogeneity of study designs in terms of type and level of soy/IF consumption, period of exposure, starting PSA level, and type of subjects.

Studies that yielded positive effects of supplementation included that of deVere White *et al*. [472], in which 62 prostate cancer patients consumed a genistein-rich extract (450 mg genistein daily plus 450 mg other IF aglycones), Hussain *et al*. [473] who supplied around 200 mg total IFs daily to men with rising PSA. Furthermore Spentzos *et al*. [474] who placed 18 prostate cancer patients with rising PSA on a low-fat diet, and a soy-protein supplement (114 mg IF daily) as well as Dalais *et al*. [475] who used a high-soy bread (117 mg IF aglycone/day) diet observed also positive effects.

In contrast to these results, a number of other studies report no changes in PSA levels following various levels and types of soy supplementation [476 – 480].

Changes in circulating androgen and estrogen levels are also often used as surrogate endpoints in intervention studies. As for PSA levels, some interventions report that soy may have favourable effects on levels of certain hormones [477, 481, 482], but others report no beneficial outcomes [473, 479].

Overall, despite some suggestions of protective effects, data from both epidemiological and intervention studies are inconsistent in regard to soy consumption, IF exposure, and prostate cancer risk.

6.4.3 Intestinal cancer

6.4.3.1 Animal studies

A limited number of studies have investigated the effect of soy IFs on development of intestinal cancer. Genistein has been observed both to enhance and to inhibit colon cancer [483 – 485] whereas exposure to soy products containing varying amounts of IFs had either no effect on colon cancer or were observed to protect against colon cancer [447, 448, 483, 485, 486]. Both decrease in precancerous colonic lesions and decreased tumor incidence has been detected [447, 448, 483, 485, 487]. It has been suggested that aglycones might be more effective than IF glycosides [485]. This finding is not supported by other studies and overall there is modest evidence that soy IFs protect against.

6.4.3.2 Human data

Colorectal cancer (CRC) is the fourth commonest cancer in the world both in incidence and mortality, with about 875 000 cases *per* year – 8.5% of all new cancer cases. In the developed world, particularly Europe, North America, Australia, and New Zealand, CRC is ranked second (behind lung cancer in men and breast cancer in women). Colon and rectal cancers exhibit different sex distribution. Colon cancer has a similar incidence in male and females whereas the incidence of rectal cancer is higher in males. There is strong evidence from epidemiological studies showing that diet plays an important role in most large bowel cancers, implying that it is a potentially preventable disease. The precise dietary components that influence CRC risk have not been fully elucidated. Epidemiological studies suggest that high intakes of fat, meat, and alcohol increase risk, whereas vegetables, cereals, and fibre decrease the risk [488].

The possible protective action of soy products on CRC is based on the following premises:

(i) Vegetarian diets, which often include soy, appear to be associated with a lower risk of CRC.

(ii) Populations of Asian countries, such as Japan and China, which consume large amounts of soy products have a low incidence of CRC.

(iii) The incidence of CRC is increasing in Japan as the diet becomes increasingly "Westernized" resulting in a decreased consumption of vegetables, carbohydrate-based foods, soy products, and increased intake of fat.

(iv) Soy contains a number of substances with potential anticancer activity, *e.g*., nondigestible oligosaccharides, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, and isoflavonoids.

Much attention has focused on the IFs (genistein, daidzein and to a much lesser extent, glycitein) in soy, which have been found to act as estrogen agonists and antagonists. These phytoestrogens are thought to have anticancer activity toward hormone dependent cancers such as breast and prostate cancer. Although CRC is not considered to be such a cancer, the human gut is known to contain ERs (ERβ) [489] and there is some evidence to suggest that these play a role in tumor development. In addition, hormone replacement therapy is associated with a modest decrease in risk of CRC (RR 0.72, 95%CI 0.48 – 1.12) [490].

Several case control, one cohort and three ecological studies have been conducted, mainly in South East Asian populations, which explored the association between soy intake and risk of CRC [491, 492]. The studies have many limitations – soy was not always the main objective of the studies, so the veracity of the soy intake data may be questionable. In addition, the time period for assessing cancer prevention was inappropriate and the adjustment for potential confounding factors was inadequate. Although an

inverse trend was suggested, nearly all of the confidence intervals overlapped and there were no significant negative associations between intake of soy (in various forms – soybeans, tofu, bean sprouts, bean curd), and colonic cancer [492]. In six of the studies, a decreased risk of rectal cancer associated with unfermented (but not fermented) soy products was reported. Conversely, in some of the studies, certain types of soy products, in particular fermented products such as miso, were associated with an increased risk of colon or rectal cancer [493 – 495]. Two studies of colorectal polyps [496, 497] showed no significant protective effects of soy.

In spite of the lack of significant effects, the general negative trend between high soy consumption and CRC risk seen in the studies to date indicates that further studies utilizing more robust methodology are warranted.

6.5 Soy IFs and menopausal symptoms

International differences in incidence of hot flushes in menopausal women, with low levels in Japan and China, together with data from prospective and cross-sectional studies from Asian countries [498, 499] suggesting that soy intake is negatively correlated with the number of hot flushes, have resulted in significant research investigating the potential role of soy and its IFs in reducing menopausal symptoms. In a case-control study, there was a trend toward a decrease in hot flushes with increased intake of IFs, although this did not reach statistical significance [500]. The lowest quartile of intake ranged up to 35 mg/day with no comparison of intakes akin to levels of exposure in Europe (<5 mg), and therefore these women may not have gained any additional benefit from their high baseline habitual intake.

Numerous short-term studies have attempted to evaluate the effect on menopausal symptoms, using a range of IF supplements, traditional soy foods or IF enriched soy foods. These intervention studies, conducted in both peri- and postmenopausal women have generated variable results, with many trials of poor quality and the soy IF supplement studies have been the subject of two recent systematic reviews [501, 502]. Although 11 published trials compared soy IFs to placebo, only 6 of these randomized controlled trials met the criteria for inclusion in the Nelson *et al*. [502] meta-analysis. The available data are contradictory for soy IFs, even amongst the larger better quality studies. These findings are consistent with previous meta-analyses [503, 504]. In the other recent systematic review [501], different inclusion criteria were considered, and the overall findings were slightly more positive, suggesting that IF supplementation may produce slight to moderate reductions in the number of hot flushes and the benefit may be more apparent in women who experience a high number of flushes. The available data therefore clearly show the need for further robust trials to identify potential effects of soy on menopausal symptoms but available data do not support a clear clinical benefit. Other forms of soy IF have also been reviewed, including soy powders, flour, and other foods but the variability in design, composition, and dose used has precluded a meta-analysis of the available trials [5].

Most of the performed studies have been short term and the question of whether consuming phytoestrogen-rich diets prior to entering the menopause would be more effective is unknown. This is more akin to the Japanese experience, where women have consumed IFs over the life course.

6.6 Soy IFs and bone health

Although data from rodent studies clearly demonstrate that soy IFs are effective in reducing bone loss and increasing bone formation, two long-term studies using ovariectomized monkeys have failed to show an effect of soy IFs on bone [505]. It is possible that the responsiveness to bone may differ between species as it is well established that there are significant species differences in the metabolic handling of IFs [506, 507].

Epidemiological evidence is supportive of a role for IFs in preventing bone loss since the incidence of hip fractures is lower in Asia than in most Western communities [508]. These differences in osteoporosis-related fractures may be accounted for by other factors including, for example, skeletal size [509, 510]. To date the available data from observational studies and short-term intervention trials have produced variable results and evaluation of the existing data is complex, given the differences in study designs, sources of IFs, dose administered and endpoints measured. The human data have recently been reviewed [511, 512]. These data suggest that when soy foods containing significant levels of IFs are substituted in the diet of postmenopausal women, bone resorption is reduced [511, 512]. There also appears to be a threshold of intake required for a measurable change in bone mineral density. These data are suggestive of beneficial effects on biochemical markers of bone turnover. Whether these data translate into long-term effects on bone density or, more importantly, fracture risk remains to be established. More long-term studies are therefore required, with fracture as an endpoint measure to determine effective doses and relative importance of IFs for potentially preventing osteoporosis.

6.7 Soy IFs and cognition

Although IFs cross the blood–brain barrier [513 – 515] and improve cognitive performance in animal models [515, 516], to date there are few well-conducted randomized control trials examining their effects in human at different stages of development.

Previous data from an epidemiological study suggested a positive association between tofu consumption and cognitive decline in middle aged Japanese-Americans with a

dose dependent increase of up to 2.8-fold in risk of developing vascular dementia when 2 – 3 or more servings of tofu were consumed *per* week [517]. Although age, education, and history of prior stroke explained 27.8% of the variance in cognitive function test scores, tofu intake only accounted for 0.8% [517]. To date, human intervention studies investigating the effects of IFs on cognitive function have been equivocal; the largest study, a double blind randomized placebo controlled trial in postmenopausal women observed no effect following a year intervention of 99 mg IFs (aglycone equivalents) on a range of measures of cognitive function [302]. In three other smaller intervention studies in postmenopausal women, there was a suggestion of beneficial effects on cognitive function following intervention with soy IF supplements. In one short-term study, postmenopausal women fed 60 mg IFs as a soy supplement showed improvements in cognitive performance following the intervention [518]. In addition, in a 6-month study the use of soy supplements providing 110 mg of soy IFs daily (given as 55 mg twice *per* day) had favourable effects on cognitive function, particularly verbal memory [519]. In the third crossover study, 60 mg/day over 6 months improved cognitive performance and mood [520]. A small randomized study was suggestive of a significant influence of IF intake on cognitive function in a group of young volunteers and showed gender differences in cognitive ability [521, 522], but time of day effects on cognitive function were not investigated [523]. Further research is required to clarify the effect of different doses and types of soy foods and to determine the relative importance of gut metabolism in affecting cognitive function.

7 Concluding remarks

The primary IF sources are soy and soy-based food items although also the impact of hidden sources of soy, *e.g.*, added soy protein isolate, soy concentrate or soy flour to bakery, meat, and vegetable products should not be neglected.

There is no consensus on which source of IFs results in the highest IF bioavailability and published studies present different results. It is known that the bioavailability of IFs is influenced by numerous factors like food composition (content of protein, fat, and dietary fiber), the food matrix (solid/liquid) as well as the chemical form (glycosides and aglycones) which might interfere with each other.

A diverse range of analytical methods has been developed and evaluated for determination of the IF content in raw plant material, foods, and food supplements, as well as for investigation of IF concentrations in biological fluids and tissues. The development of analytical methods has facilitated insight into their fate in living organisms (ADME). At the same time intensive research using *in vitro* systems has elucidated the mechanism of action of soy IFs on cellular levels. Comprehensive data have been generated on their ER mediated mechanism of action and their estrogenic potency, antioxidant, and anti-inflammatory activity, effects on angiogenesis, and protection against genotoxic effects *via* up-regulation of phase II enzymes. A new discipline, nutrigenomics, has drawn attention to genetic polymorphism as determinant of biological response to soy IFs.

IF-rich soy foods, soy protein isolate, and IF extracts have a variety of effects that may influence cardiovascular risk. Animal studies and investigations in healthy human subjects indicate beneficial effects of substitution of animal protein with soy protein containing IFs on blood lipids as well as on development of atherosclerosis in animal models. IFs alone fed as pure compounds or extract have been found to have no or little lipid lowering effect indicating that the mechanism of action of IFs on lipids is complex and probably involves an interaction with the food matrix. Also, other mechanisms that mediate the development of atherosclerosis may be influenced by dietary IF intake, such as LDL oxidation, vascular inflammation, platelet aggregration and endothelium-dependent vasodilation. Vasodilatory effects of soy IFs suggest a positive effect on arterial function and there is persuasive evidence from studies from nonsclerotic animal arteries. The effect of soy IFs on arterial function in atherosclerotic animal models is less clear. Also data from human studies remain sparse and conflicting and it is not yet possible to draw conclusions regarding effects of soy IFs on endothelial function. Finally, individual response to dietary IFs may depend on ER genotype with regard to biomarkers of cardiovascular risk.

The effects of soy IFs on the proliferation of breast cancer cells *in vitro* have been documented, and the compounds have been shown to affect tumor invasion *in vitro* as well as to inhibit angiogenesis. Although several animal studies confirmed cancer preventing effects of a soy containing diet in laboratory animals, more recent studies demonstrate increased incidence of mammary tumors in laboratory animals genetically predisposed to mammary cancer (transgenic animals) or those with chemically induced mammary carcinogenesis fed diet added IF rich soy extracts. Furthermore, animal studies provide evidence that time of exposure to soy IFs is crucial for their effects on breast cancer. Epidemiological studies investigating the relationship between soybean intake and breast cancer in Asian women suggest an inverse association for both premenopausal and postmenopausal breast cancer whereas the association for Caucasian women is not apparent. In Western countries, the dietary exposure to soy IFs is traditionally low but it may drastically increase with use of IF rich supplements during menopause as the soy IF containing supplements are heavily marketed as an alternative to hormone replacement therapy that recently has been shown to have serious adverse effects. The estrogenic potency of IFs is weak compared to estradiol, but their biological potential cannot be ignored as blood levels of these compounds can exceed endogenous

estradiol concentrations by the factor 10 000 following the change of dietary habits. Therefore, the increased use of IF rich supplements should be regarded with caution and their recently reported carcinogenic effect in laboratory animals should be further elucidated.

A variety of *in vitro* studies have demonstrated that IFs can inhibit growth of human prostate cancer cell lines, and cell lines derived from normal human prostatic epithelium. There is a considerable evidence to suggest that IFs can modulate a wide range of signaling cascades controlling prostate cell growth, proliferation, invasion, and angiogenesis. Animal studies provide evidence that genistein and soy protect against prostate cancer. Despite some suggestions of protective effects, data from both epidemiological studies and intervention studies are inconsistent in regard to soy consumption, IF exposure, and prostate cancer risk.

A limited number of animal studies have investigated the effects of soy IFs on development of intestinal cancer. Exposure to soy products containing varying amounts of IFs had either no effect on colon cancer or was observed to protect against colon cancer. In some human studies, the general negative trend between high soy consumption and CRC risk was seen. Further studies utilizing more robust methodology are warranted.

Due to their estrogenic activity soy IFs received considerable attention as an alternative to hormone replacement therapy. Numerous short-term studies, which have attempted to evaluate the effects on menopausal symptoms have generated variable results with regard to hot flushes. The benefit may be more apparent in women who experience a high number of flushes.

Epidemiological evidence is supportive of a role of IFs in preventing bone loss. The human studies suggest that when the soy foods containing significant levels of soy IFs are substituted in the diet of postmenopausal women, bone resorption is reduced.

To date there are few well-conducted randomized control trials examining the effect of soy IFs on cognitive function. Human intervention studies have been equivocal. Further research is required to determine the effectiveness of soy IFs in relation to cognitive function.

In conclusion, many questions remain surrounding soy and soy IFs with regard to impact on health, reduction of disease risk, and improvement of quality of life. At the same time, several promising research outcomes aroused the interest of the food and pharmaceutical industry in the production of food components, dietary supplements and pharmaceuticals. It should be noted that, epidemiological evidence of beneficial health effects is based on communities where exposure to IFs starts *in utero* and continues from birth throughout life as consumption of soy and IF containing foods. How an increased dietary exposure to soy and IFs later in life affect individuals with traditionally low intake of these food components is not known at the moment. It even might be a cause for concern. Thus, further elucidation of mechanisms of action, human studies and safety evaluation of these compounds is a challenge for the future. At the same time, an important consideration is that foods rich in IFs are also a part of the healthy diet, *i.e.*, low in fat, high in fibre, rich in vegetables and fruits and moderate in animal protein, and that a healthy diet is a part of a healthy lifestyle, which can certainly help to decrease risk of disease. Further research is needed in order to fully explain how these food components contribute to improve health.

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